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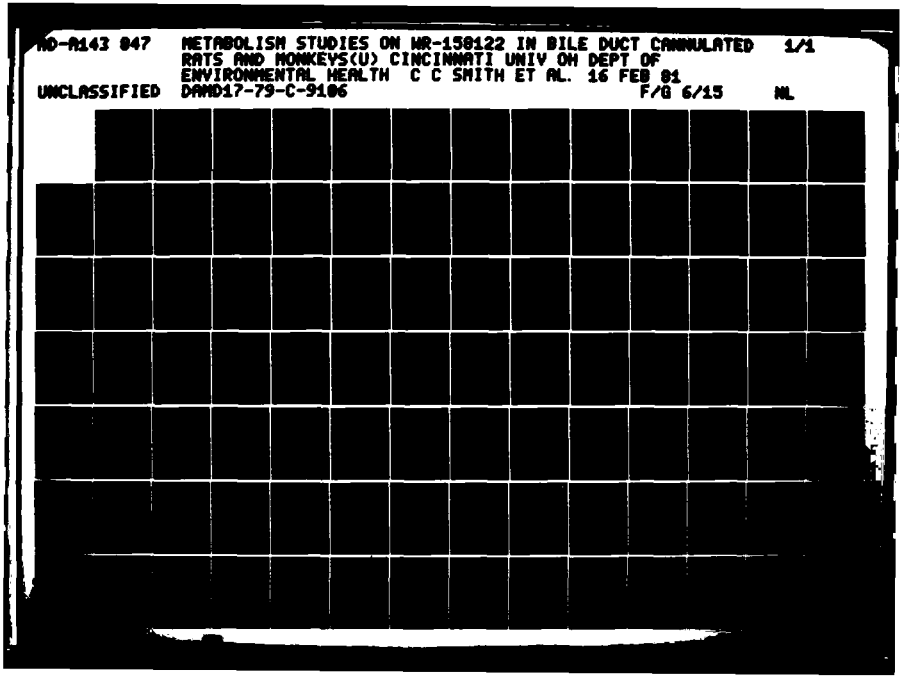

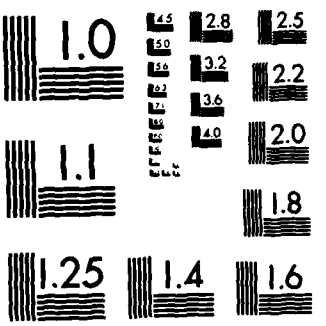

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Annual Report

Metabolism Studies on WR-158,122 in Bile Duct Cannulated Rats and Monkeys

Carl C. Smith, Ph.D.
M. Wilson Tabor, Ph.D.
Geraldine F. Wolfe, M.S.
Steele F. Mattingly, D.V.M.
David H. Bauman, D.V.M.
Gary L. Keller, D.V.M.

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Department of Environmental Health and
Department of Laboratory Animal Medicine
University of Cincinnati College of Medicine
Cincinnati, Ohio 45267

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Section 1: We have developed a procedure for preparing uniform fecal samples for LSC counting and study of fecal metabolites. The procedure requires milling of a rat or monkey feces sample with anhydrous Na_2SO_4 in a stainless steel ball mill. The fine off-white flours that result are passed through a fine wire sieve (18-8 stainless steel wire, 30 holes/inch). This sieving increases homogeneity by removing traces of unground material and dietary fiber. Digestion of samples with NaOH and counting in dioxane-toluene-

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naphthalene with Cab-O-Sil (DTN-C) gives reproducible recoveries. This procedure was adequate for 0.25 g and 0.5 g samples but inadequate for 1.0 g samples. In a study of 7 solvent/digestants we concluded that the best solv/dig were 1) Soluene, 2) NaOH and 3) Protosol. These three solv/dig extracted the most dpm from feces samples and showed the best reproducibility for 0.25 g aliquots. Protosol and Soluene digests count just as efficiently in Biofluor as in DTN-C. Ten replicate Soluene digests counted in Biofluor and 10 replicate NaOH digests counted in DTN-C showed mean % recoveries which matched well (NaOH, 40.6 $\pm$ 0.32 SE; Soluene, 41.3 $\pm$ 0.43 SE). Recovery of ^{14}C by combustion assay was compared with results obtained by digestion in NaOH, Soluene, and perchloric acid-H_2O_2. The combustion assays gave recoveries that agreed well with values obtained by our digestion procedures. When 1.0 g aliquots of 4 milled feces samples were extracted sequentially 3 times with THF and digested once with Soluene, the THF extraction recoveries matched the digestion methods for monkey feces. Recovery of ^{14}C in rat feces samples was 10 to 20% lower than digestion assays. Six feces samples were extracted sequentially with benzene, ethyl acetate, acetonitrile and tetrahydrofuran to see whether this procedure would give complete recovery of fecal ^{14}C. 96% of total dpm's were recovered in the total ethyl acetate and benzene extracts for all the samples except the non-operated rat CT-4. In this rat only 78 to 82% was extracted by these solvents, suggesting that the metabolites in this rat's feces are different from those in monkey 75-5 ♀ (bile-duct cannulated). Most of the undissolved fraction in rat CT-4 was soluble in acetonitrile. Further work is planned to find the best series of solvents for sequential extraction and separation of fecal ^{14}C containing metabolites. This will be followed by purification of the metabolic fractions on HPLC and eventual structural studies using GC-MS and other analytical techniques such as IR and NMR if sufficient material can be generated.

Section 2: Blood levels, excretion and tissue distribution of WR-158,122 ^{14}C were studied in 10 bile duct ligated rats, 10 bile duct cannulated rats, 8 control rats and in 2 control and one bile duct cannulated monkeys. In 72 hours bile duct ligated rats excreted about 16.3 $\pm$ 3.8% as ^{14}C in the urine and the remaining ^{14}C was recovered primarily in the feces. About 2% was recovered in the carcass and tissues at necropsy. Bile duct cannulated rats excreted about 10.9 $\pm$ 6.1% in the bile, 2.3 $\pm$ 1.3% in the urine and the remainder in the feces. Only about 1.3 $\pm$ 1.0% was recovered in the carcass and tissues. Control rats excreted the compound rather rapidly, 1.5 $\pm$ 0.8% of the ^{14}C was recovered in the urine, 0.3 $\pm$ 0.2 in the carcass and other tissues and the remainder in the feces. Total average recovery (as ^{14}C) in the three groups of rats varied from 94 to 100% of the dose. Two experiments were conducted in a single bile duct cannulated monkey. In the first the blood and plasma levels were highest at two hours after dosing. Plasma levels routinely exceeded levels in whole blood indicating little sequestration of WR-158,122 (as ^{14}C) in the red cells. Both levels were low at 24 hours and below detection at 48 hours. In this bile duct cannulated monkey biliary excretion over a 96 h. period accounted for 0.6 to 0.9% of the dose (as ^{14}C), urinary excretion for 3.1% and 87 to 95% of the dose was recovered in the feces. In two control monkeys urinary excretion over a 96 hour period amounted to 14 and 18%; 70% of the dose (as ^{14}C) was recovered in the feces. Excretion was essentially complete after 96 hours. In both rats and monkeys WR-158,122 appears to be rapidly but incompletely absorbed. Rats excreted 10% more in the bile but only 2-3% in the urine except in the case of bile duct ligated rats which excreated about 16% of the dose (as ^{14}C) in the urine.

Block 19 - Key Words

feces
homogenization
ball-mill
extraction
digestion
organic solvents
rat
monkey
bile duct cannulation
bile duct ligation
urinary excretion
biliary excretion
fecal excretion
LSC and ^{14}C and metabolites
WR-158,122
sulfonyl quinazoline
2,4-diamino-6-(2 naphthylsulfonyl) quinazoline

A-1

TABLE OF CONTENTS

DD Form 1423

Table of Contents

DD Form 1423

TABLE OF CONTENTS

TABLE OF CONTENTS

SECTION 1. A NEW PROCEDURE FOR PREPARING UNIFORM FECAL SAMPLES FOR LSC

1.1 Introduction

Pursuing the identity of the fecal products following the oral (or parenteral) administration of WR-158,122-^{14}C posed technical problems. We were unaware of a method of homogenizing (and digesting) feces that did not require chemical and probably degradative action on the sample. In other words how can one get homogeneous fecal samples without changing the composition of the feces themselves? Standard methods including homogenization with acid or alkali or solvent extraction (MeOH) after ~~dessication~~ desiccation did not appear satisfactory for our needs; therefore, we began the development of a new procedure.

1.2 Preliminary Experiment

Our first experiment consisted of digesting fecal samples (ground with mortar and pestle using various solvents.

Feces Sample

The first sample, spiked rat feces (SRF), was prepared by grinding 6.0 g of control rat feces with 60 g of anh. Na_2SO_4 and 0.5 ml of WR-158,122-^{14}C treatment suspension with a mortar and pestle and subsequently ball-milling this. The final milled sample was calculated to contain 8,518 dpm/g.

The second sample (BL-10-24) was prepared by grinding the 24 hr feces sample from bile-ligated rat #10 with anh. Na_2SO_4 (3.78 g feces + 20 g Na_2SO_4).

Solvent/Digestants

1) Dimethylsulfoxide (DMSO)

2) DMSO: tetrahydrofuran (THF), (1:1)

3) THF

4) Protosol®*

*Registered trademark for a tissue solubilizer, obtained from New England Nuclear, 549 Albany St., Boston, Massachusetts 02118.

5) Soluene 100 ®*

6) Tetramethylammonium hydroxide 24% in methanol (TMH)

Procedure

Samples (0.25 g) were weighed into LSC vials, 1 ml of each of the above solvent/digestants was added and the vials placed in a 70°C incubator. Because digestion appeared incomplete at 24 hr we added 0.5 ml of 0.5 N NaOH and 0.5 ml of tertiary butyl hydrogen peroxide (TBHP) bleach to each sample and incubated an additional 10 hr. Samples were counted after vigorous shaking with 15 ml of a counting medium consisting of dioxane, toluene, naphthalene plus 5% Cab-O-Sil ®** (DTN-C) and the counts corrected for quenching with an internal standard.

Results

As seen in Table 1, recovery of fecal ^{14}C in dpm/g varied from 8730 to 9790 for SRF and from 70,800 to 90,000 for the BL-10-24 sample; the lowest value for the first sample was obtained with TMH, the lowest for BL-10-24 with DMSO-THF. This unacceptable variability suggested we were encountering either 1) sample inhomogeneity, 2) incomplete digestion or 3) poor gel formation in the counting vials.

Table 1.1

Recovery of Fecal ^{14}C in Various Solvent/Digestants

Solvent/ Digestant	SRF dpm/g***	BL-10-24
DMSO	8740	89,200
DMSO-THF (1:1)	9790	70,800
THF	9170	78,500
Protosol	9650	86,400
Soluene	9460	83,900
TMH	8730	90,000

*Registered trademark for a sample solubilizer obtained from Packard Instrument Company, Inc., 2200 Warrenville Rd., Downers Grove, Illinois 80515

**Registered trademark for thixotropic agent (Silicon dioxide), available at Cabot Corp., 21010 Center Ridge Rd., Rocky River, Ohio 44116

***Gram of final milled powder

The next step was to develop a better fecal homogenization technique. In 1936 one of us (C.C.S.) had used a ball mill to prepare powdered dehydrated muscle samples. Using a stainless steel ball mill* loaned to us by Dr. Wm. E. Kuhn, Dept. of Material Sciences and Metallurgical Engineering, Univ. of Cincinnati, we found that the best sample preparations from rat and monkey feces were obtained by grinding the feces with 4 to 8 parts anh. Na_2SO_4 for 30 to 60 min. using a combination of ½ and 1 inch stainless steel or chrome plated steel balls. The final preparations were fine, off-white flours which appeared homogeneous. Later (Exper. 5 and after) all flours were passed through a fine wire sieve (18-8 stainless steel wire, 30 holes/in.) which removed traces of unground material and dietary fiber. With almost no changes this is the procedure we have adopted for preparing fecal samples.

*Modal 611 unlined steel grinding jar, Jar size 0 (0.50 gal), Norton Chemical Process Products Division, Akron, Ohio 44309.

1.3 EXPERIMENT 1

Introduction

From the preliminary experiment we concluded that NaOH may be as good a digestant as any of the others we had tried and could be counted adequately in DTN-C. Therefore, we used NaOH in the next experiment.

Purpose

To study the linearity and reproducibility of alkali-digested milled feces.

Sample Preparation

The feces used were the 24 and 48 hr samples from 6 bile-duct ligated (BL) rats treated with 10 mg/kg oral doses of WR-158,122-^{14}C. They were weighed and milled with known amounts (4 to 8 parts) of anh. Na_2SO_4 in a stainless steel ball mill for 30 to 60 min.

Procedure

The milled feces aliquots (0.25, 0.5 or 1.0 g weighed on an analytical balance) were put in LSC vials and 1 ml of 1 N NaOH was added. After 24 hr 1 ml of H_2O was added to 0.5 g and 1.0 g samples to increase the liquid volume and assist digestion. After 24 hr the oven temperature was increased from 70°C to 80°C and incubation was continued to 72 hr. Glass beads were placed in some vials to assist in suspending sample adhering to vial walls. The recovery of fecal ^{14}C was expressed as % dose.

Results

The figures in Table 2 indicate that NaOH digestion followed by counting in DTN-C worked adequately for 0.25 and 0.5 g samples of these milled feces. The high results obtained with some of the samples suggested that the procedure is inadequate for counting 1 g samples. The difference among 6 duplicate 0.25 g samples varied from 1 to 5%. For 0.5 g samples the variation

was from 1 to 11%. The 1 g samples were occasionally higher (4/12) or lower (2/12) or similar (6/12) to the values we obtained for the 0.25 and 0.5 g aliquots of the same sample. The higher apparent recovery values for certain 1 g aliquots were due to abnormally low estimates of efficiency using the internal standard method. For this reason there appear to be advantages in restricting the sample size to 0.25 g (or possibly 0.5 g).

Table 1.2

Reproducibility and Linearity of Alkaline Digests of Milled Rat Feces in % Dose

Wt. of milled feces (g)	BL-9		BL-10		BL-11	
	24*	48*	24	48	24	48
0.25	78.8	5.2	82.3	10.8	56.1	4.7
	79.4	5.1			58.7	4.5
0.5	77.0	5.4	71.7	10.5	59.7	4.9
	76.7	5.4	79.8	10.9		
1.0	88.3	5.3	68.0	10.7	68.8	5.1

Wt. of milled feces (g)	BL-12		BL-13		BL-14	
	24	48	24	48	24	48
0.25	69.8	11.0	71.2	6.4	65.3	14.6
					65.6	14.4
0.5	67.0	10.5	72.9	6.4	70.9	15.0
			72.8	6.6		
1.0	58.7	10.6	86.9	6.0	98.1	14.0
	56.1	10.6				

*24 and 48 hr samples.

1.4 EXPERIMENT 2

Introduction

In Experiment 1 we proved that 1.0 N NaOH is a good digestant for fecal samples. We also established that it is best to limit sample size to 0.25 g samples. In this study we digested 0.25 g samples of 5 milled feces in a series of 7 solvent/digestants.

Purpose

To find out which of a series of solvent/digestants extracts the most dpm from milled feces samples and does so most consistently. To check reproducibility of 0.25 g samples.

Sample Preparation

Five feces samples prepared by milling with 4-8 parts anh. Na_2SO_4 in a stainless steel ball mill were used.

1) SRF as described on page 1

2) BL-10-24. 24-hr feces from bile-duct ligated rat #10 treated with WR-158,122-^{14}C (10 mg/kg oral dose).

3) CT-4-48. 48-hr feces from a non-operated rat treated with WR-158,122-^{14}C (10 mg/kg oral dose).

4) Spiked monkey feces (SMF). Control monkey feces (39.14 g) from rhesus 75-5 were ground with 123.13 g of anh. Na_2SO_4 and 4.0 ml of WR-158,122-^{14}C treatment suspension with a mortar and pestle and subsequently were ball-milled. The final milled sample was calculated to contain 27,250 dpm/g.

5) 75-5-48. 48-hr feces sample from bile-duct cannulated rhesus 75-5 ♀ treated with WR-158,122-^{14}C (5 mg/kg oral dose).

Solvent/Digestants

1) DMSO

2) DMSO-THF (1:1)

3) THF

4) Protosol

5) Soluene

6) Tetramethylammonium hydroxide (TMH)

7) NaOH, 0.5 N

Digestion Protocol

a. Weigh milled feces samples (approximately 250 mg) on an analytical balance and place in 20 ml LSC vials.

b. Add 1 ml of solvent/digestant.

c. Digest in oven set at 68-70°C for 24-48 hr.

d. Bleach with 0.5 ml tertiary butyl hydrogen peroxide (TBHP) at 2-24 hr.

e. Add either 15 ml of 5% DTN-Cab-O-Sil (DTN-C) or 15 ml of Biofluor.

f. Cool and dark-adapt samples at least 2 hr at 4°C before counting.

g. Internally standardize with toluene ^{14}C, if desired.

Procedure

In this experiment digestions were carried out as described in the Digestion Protocol except incubation was terminated at 24 hr and all samples were counted in 15 ml of DTN-C.

Results

Total ^{14}C recovery expressed as % dose in Table 3 showed total ranges shown below:

	% dose range	Coefficient of Variation, %
SRF*	95.1-103.2	8.1
BL-10-24	71.1-84.2	16.8
CT-4-48	5.1- 6.7	26.7
SMF*	97-102.3	5.3
75-5-48	35.4-38.9	9.4

*Calculated to a mean recovery of 100%.

In Table 4 we have listed the results we obtained with each solvent/digestant (solv/dig) with symbols (L, l, =, h, H) and in parentheses, the differences between each pair of duplicates expressed in percent variation to make them comparable. On the basis of these two criteria and giving major weight to the solv/dig which gave the best recoveries (most H or h, least l or L) we classified the solv/dig as "good" or "poor". Thus, we classified Protosol, Soluene and 0.5 N NaOH as "good" and the rest as "poor".

Discussion

An undissolved salt residue was present in all the solv/dig except 0.5 N NaOH which dissolved most of the salt. We are uncertain whether this salt residue interferes with LSC. Bleach was effective in all the solv/dig except TMH which bleached poorly. There were traces of brown particles in many of the digested samples. These may represent "hot spots" of undigested feces and/or drug. All the samples formed good gels except THF which required the addition of 0.5 ml 0.5 N NaOH.

Conclusions

From our evaluation of this series of solvent/digestants we concluded that the best were 1) Soluene, 2) NaOH and 3) Protosol.

Table 1.3

Extraction of Milled Feces with a Series of Solvent/Digestants

Solvent/Digestant	SRF*	BL-10-24	CT-4-48	SMF*	75-5-48
	Total ^{14}C in % of Dose				
DMSO	95.3	83.3	5.9	100.6	36.7
	101.5	78.4	6.1	100.9	37.7
DMSO-THF (1:1)	97.7	75.6	5.8	101.7	37.3
	98.5	78.1	5.5	100.4	37.8
THF	101.1	75.5	5.1	102.0	37.9
	102.2	76.3	6.1	98.5	36.9
Protosol	102.2	84.2	6.4	102.0	37.4
	103.2	75.0	5.8	99.1	37.2
Soluene	99.8	78.5	6.0	98.8	37.8
	102.4	79.3	6.4	97.8	38.2
TMH	98.6	74.6	6.7	97.2	35.7
	95.1	71.1	5.9	97.0	35.4
NaOH	99.8	78.7	6.1	102.3	38.9
	102.4	82.6	6.2	101.5	38.8

*Calculated to a mean recovery of 100% for all 7 samples.

Table 1.4

Classification of Solvent/Digestants Based on Recovery of Fecal ^{14}C From Five Milled Feces Samples

Solvent/ Digestant	SRF	BL-10-24	CT-4-48	SMF	75-5-48	Classification
DMSO	L (6.2)	H (4.9)	= (0.2)	h (0.3)	l (1.0)	Poor av. 2.52
DMSO-THF	L (0.8)	l (2.5)	l (0.3)	h (1.3)	h (0.5)	Poor av. 1.08
THF	= (1.1)	L (0.8)	L (1.0)	= (3.5)	= (1.0)	?Poor av. 1.48
Protosol	H (1.0)	H (9.2)	= (0.6)	= (2.9)	= (0.2)	Good av. 2.78
Soluene	h (2.6)	h (0.8)	h (0.4)	l (1.0)	H (0.4)	Good av. 1.04
TMH	L (3.5)	L (3.5)	H (0.8)	L (0.2)	L (0.3)	Poor av. 1.66
NaOH(0.5 N)	h (2.6)	H (3.9)	= (0.1)	H (0.8)	H (0.1)	Good av. 1.5

L = lower than mean recovery figure

l = slightly lower than mean recovery figure

= equal to mean recovery figure

h = slightly higher than mean recovery figure

H = higher than mean recovery figure

() = difference between duplicate samples

1.5 EXPERIMENT 3

Introduction

Previously we had counted feces digests primarily in DTN-C. This is a good medium but often results in very solid gels which are tedious to use and to internally standardize. Therefore we decided to try a different medium.

Purpose

To determine whether the counting efficiency of Protosol and Soluene digests of milled feces in Biofluor ®* equals that of NaOH digests.

Samples

The four milled feces samples used in Experiment 2 were employed: 1) SRF, 2) SMF, 3) CT-4-48 and 4) 75-5-48.

Solvent/Digestants

1) Protosol

2) Soluene

Procedure

See Digestion Protocol.

Results

The results in Table 5 indicate that for these four milled feces samples, the values obtained with Protosol and Soluene in Biofluor were essentially the same and had the same variability as those obtained using NaOH in DTN-C. Standard deviations varied from 0.3 to 2.2. If we expressed these figures as coefficient of variation (in %), the values ranged from 2 to 7%, which we considered acceptable.

Conclusions

We can employ Biofluor for future assay work with Protosol and Soluene digests. This circumvents the need to prepare the more time consuming DTN-C medium.

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*Registered trademark for a liquid scintillation counting fluid obtained from New England Nuclear (NEN), 549 Albany Street, Boston, Massachusetts 02118.

Table 1.5

Comparison of Two Media for Counting Feces Digests

Feces Sample	Solvent	LSC MEDIA Biofluor	5% DTN-Cab-O-Sil
SRF*	Protosol	100.1, 99.9	99.6, 100.5
	Soluene	97.3, 102.7	98.7, 101.3
		100.0 ± 2.2**	100.0 ± 1.12
CT-4-48	Protosol	5.7, 6.7	6.4, 5.8
	Soluene	6.0, 6.0	6.0, 6.4
		6.23 ± 0.46	6.15 ± 0.30
SMF*	Protosol	99.8, 100.2	101.4, 98.5
	Soluene	100.3, 99.7	100.5, 99.5
		100.0 ± 0.29	100.0 ± 1.25
75-5-48	Protosol	38.7, 38.4	37.4, 37.2
	Soluene	39.5, 38.8	37.8, 38.2
		38.8 ± 0.47	37.7 ± 0.44

*Calculated to a mean recovery of 100% for all four samples.

**Mean ± S.D.

1.6 EXPERIMENT 4

Introduction

Having established in Experiment 3 that Biofluor is a good medium for Soluene digests of milled feces samples we decided to compare % recovery of 10 Soluene digests in Biofluor medium with % recovery of 10 NaOH digests in DTN-C medium.

Purpose

To compare the reproducibility of NaOH and Soluene digests of a milled feces sample.

Sample

The 48 hr milled feces sample from bile-duct cannulated monkey 75-5 ♀ given a single 5 mg/kg oral dose of WR-158,122-^{14}C.

Solvent/Digestants

1) 0.5 N NaOH

2) Soluene

Digestion Procedure

The protocol was modified as follows: Samples were incubated 48 hr. NaOH digests were counted in 15 ml of DTN-C, Soluene digests in 15 ml of Biofluor.

Results

The 10 replicate NaOH digests showed a mean % recovery of 40.6 ± 1.0 SD. The 10 replicate Soluene digests showed a mean % recovery of 41.3 ± 1.35 SD (see Table 6). Thus the results with the two types of digests were quite comparable. The average counting efficiency of the 10 NaOH digests was 81.6%; for the 10 Soluene digests the efficiency was 82.6%.

Discussion

Although the 10 replicates for each solvent match well in this experiment, we noted that some earlier recovery figures for this same feces sample were a

bit discrepant.

Exp. #2	NaOH (DTN-C)	38.9 38.8	Exp. #3	Soluene (DTN-C)	37.8 38.2
			Exp. #3	Soluene (Biofluor)	39.5 38.8
Exp. #4	NaOH (DTN-C)	40.6 ± 1.0	Exp. #4	Soluene (Biofluor)	41.3 ± 1.35

Although the variability was only 1.7 to 1.8% for NaOH and 1.8 to 3.5% for Soluene, several questions remained to be answered. How do these values compare with the results that would be obtained by combustion assay of the same samples? Our second question was: Are the milled samples really homogeneous? To answer this latter question in part we sieved all of the previously milled samples using a stainless steel wire screen sieve (18-8, 30 holes/inch). This usually removed only unground fibers but in some samples there were residues which suggested incomplete milling. These latter residues were reground and mixed with the original powder. This slight inhomogeneity could possibly have contributed to our aliquot to aliquot variability but probably did not account for the differences among the the different experiments listed above. Nevertheless, passing the feces through a fine sieve is a valuable check on homogeneity of the samples and adequacy of the milling process. We have adopted it as the last step of our standard feces preparation procedure.

Conclusions

1. Sample replication of 10 NaOH digests counted in DTN-C and 10 Soluene digests counted in Biofluor is very good within the same experimental run.

2. There are variations in sample recovery values among different experimental runs which led us to question whether our digestion procedures are giving recoveries as good as those one would expect from combustion assays.

Table 1.6

Reproducibility of NaOH Digests and Soluene Digests of a Milled Monkey Feces Sample

	% Recovery (as ^{14}C)		
	NaOH Digests in DTN-C		Soluene Digests in Biofluor
1	41.6	11	43.7
2	39.2	12	40.7
3	40.7	13	41.2
4	40.1	14	40.3
5	40.3	15	41.2
6	40.7	16	39.4
7	39.1	17	40.2
8	40.5	18	40.6
9	42.1	19	42.4
10	41.7	20	43.0
	$\bar{x}$ 40.6 ± 1.0*		$\bar{x}$ 41.3 ± 1.35

*Mean ± S.D.

1.7 EXPERIMENT 5

Introduction

The variation we had observed among different experimental runs of milled feces samples led us to question whether our digestion procedures were giving recoveries comparable to those one would obtain from a combustion assay.

Purpose

To compare the results following digestion of fecal samples with NaOH, Soluene, and perchloric acid-hydrogen peroxide ("PH") of Mahin and Lofberg (1) with the results of combustion assays of the same samples.

Feces Samples (all milled)

1) 75-5-24 - 24 hr feces sample from rhesus 75-5 ♀ (bile-duct cannulated). (5 mg/kg oral dose of WR-158,122-^{14}C)
2) 75-5-48 - described on page 7
3) BL-13-24 - 24 hr feces sample from bile-duct ligated rat #13 (10 mg/kg dose of WR-158,122-^{14}C)
4) CT-4-24 - 24 hr feces sample from non-operated rat #4 (10 mg/kg oral dose of WR-158,122-^{14}C).

Solvent/Digestants

1) 0.5 N NaOH
2) Soluene
3) "PH": 60% perchloric acid* (0.3 ml) plus 30% hydrogen peroxide** (0.4 ml) (1,2).

Procedures

Combustion Assays.

The 4 milled feces samples were submitted for combustion assay to

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*60% perchloric acid - Reagent A.C.S. - Fisher Scientific Co., Chemical Manufacturing Division, Fair Lawn, New Jersey 07410.

**30% H_2O_2 - "Baker Analyzed" Reagent - J.T. Baker Chemical Co., Phillipsburg, N.J. 08865.

the New England Nuclear LSC Applications and Assay Laboratories.* Sample weights employed for the combustion assays ranged from 95 to 160 mg and samples were run in triplicate. The samples were combusted in a Packard 306B oxidizer and counted in a Packard 3385 counter at 30% gain, 50-1000 window. Results were corrected for quench by the channels ratio procedure.

Digestion

The Digestion Protocol was modified as follows:

NaOH digests were counted in 15 ml of DTN-C and Soluene digests were counted in 15 ml of Biofluor. The "PH" digests were incubated for 4 hr at 70°C and counted in 15 ml of Scintiverse®.**

Results

The combustion assays gave results which were in general agreement with values obtained by other digestion procedures (see Table 7). Most of the NaOH values compare closely with combustion values. Most Soluene values were somewhat higher than combustion values. Some of the "PH" values were slightly lower than combustion values and this might be explained by $^{14}CO_2$ loss in this digestion method, if any of the fecal ^{14}C was labile (2).

Conclusions

This experiment established that the recoveries of fecal ^{14}C by combustion and digestion procedures were very similar. These results also indicated that the digestion procedures were probably reliable and that any of the three digestants, Soluene, NaOH, or Protosol, were applicable.

The next step was to find out whether an extraction procedure would give us equally good recoveries. This was a necessary step in preparing extracts for identification of fecal metabolites. An adequate extraction procedure should extract at least 80-90% of the ^{14}C content of the feces.

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*We wish to thank Dr. Yutaka Kobayashi for his assistance with these assays.

**Scintiverse is the registered trademark for a scintillation counting fluid obtained from Fisher Scientific Co., Chemical Manufacturing Div., Fair Lawn, N.J. 07410.

Table 1.7

Comparison of Recovery of Fecal ^{14}C by Four Methods

Feces Sample	Combustion Assay*	Total ^{14}C as dpm/mg NaOH Digestion DTN-C	Soluene Digestion Biofluor	"PH" ** Digestion Scintiverse
75-5-24	84.3 ± 0.5	84.3	88.8 91.8	81.3 84.3
75-5-48	102.0 ± 2.4	106.1	108.5 112.3	105.0 107.2
BL-13-24	97.8 ± 2.0	97.8	96.7 95.6	93.3 91.4
CT-4-24	65.5 ± 1.0	64.4	68.7 62.8	65.3 63.9

* New England Nuclear, Inc.

** Perchloric acid - H_2O_2

1.8 EXPERIMENT 6

Introduction

In the preceding experiment we established that our recoveries of fecal ^{14}C by combustion and digestion procedures were reasonably similar. The next question was to find out whether an extraction procedure would provide equally good recoveries.

Purpose

To determine whether tetrahydrofuran (THF) completely extracts the total ^{14}C in milled feces samples.

Samples

The four milled feces samples (75-5-24; 75-5-48; BL-13-24; CT-4-24) used in Experiment 5 were employed in this experiment.

Solvent/Digestants

1) Tetrahydrofuran - Fisher Scientific Co. (HPLC grade)

2) Soluene

Protocol

One gram quantities of the 4 milled feces samples were placed in 15 ml graduated centrifuge tubes and extracted three times with 5 ml THF as follows: tubes were shaken for 15 min on an automatic shaker, centrifuged for 10 min and each solvent layer poured into a LSC vial. The THF was evaporated with N_2 in a Meyer N-evaporator ®* in separate LSC vials labeled extract 1, 2, and 3. Each residue was dissolved with 1 ml of THF, bleached, if necessary, with 0.5 ml TBHP, and counted in 15 ml of Biofluor.

The residues of the 1 g feces aliquots (after THF extraction) were digested with 2 ml of Soluene for 26 hr at 70°C. TBHP (0.5 ml) was added and the tubes

*Meyer N-evaporator, Model III, obtained from Organomation Assoc., Inc., Northborough, Mass. 01532.

were centrifuged for 30 min. The supernates were poured into LSC vials and counted in 15 ml of Biofluor.

Results

THF extracted almost the same dpm/mg (see Table 7) as were obtained by combustion assay or digestion by NaOH, 0.5 N, Soluene, or "PH", for the two monkey feces samples. Soluene digests of the 1.0 g residues of monkey feces samples recovered only an additional 1.8% for 75-5-24 and 3.2% for 75-5-48. However, THF extracted only 82% of total ^{14}C in CT-4-24 and 92% in the case of BL-13-24. Soluene added an additional 18% in the case of CT-4-24 and 7.8% for BL-13-24.

When Soluene digest recovery was added to THF recovery, the total recovery figures for monkey feces samples were about 7% higher than combustion figures but agreed well with the Soluene values. For the rat feces samples, the total recovery figure was slightly lower than combustion figures but probably not significantly different from the digestion values.

Conclusions

Recovery of fecal ^{14}C with THF extraction matched recovery obtained by combustion, NaOH digestion, Soluene digestion, and "PH" digestion very well for the two monkey feces samples. Recovery of fecal ^{14}C for rat feces with THF was about 20% lower than combustion recovery for the CT-4-24 sample and about 10% lower than combustion recovery for the BL-13-24 sample. This suggests that rat feces samples contain metabolites of WR-158,122 not extracted by THF.

Table 1.8

THF Extraction Compared with Combustion and Three Digestion Methods

Feces Sample → / Method ↓	Total ^{14}C in dpm/mg			
	75-5-24*	75-5-48*	CT-4-24*	BL-13-24*
THF Extract 1	79.6	92.9	46.7	76.0
2	8.5	11.3	5.4	10.5
3	0.9	1.3	0.9	1.5
Soluene digestion	1.6	3.5	11.8	7.4
Total	90.6	109.0	64.8	95.4
Combustion**	84.3 ± 0.5	102.0 ± 2.4	65.5 ± 1.0	97.8 ± 2.0
NaOH Digestion**	84.3	106.1	64.4	97.8
Soluene Digestion**	88.8	108.5	68.7	96.7
	91.8	112.3	62.8	95.6
"PH" Digestion**	81.3	105.0	65.3	93.3
	84.3	107.2	63.9	91.4

* Milled feces sample

** Results from Exp. 5

1.9 EXPERIMENT 7

Introduction

In Experiment 6 we found that THF extraction recovered as much fecal ^{14}C from monkey feces samples as did the combustion and digestion procedures. On the other hand, THF extraction of rat feces gave recoveries that were somewhat lower. We decided to do sequential extraction of several feces samples with 4 organic solvents to see whether a different extraction procedure would give us complete recovery of fecal ^{14}C.

Purpose

To determine whether sequential extraction of feces samples with four organic solvents (benzene, ethyl acetate, acetonitrile, tetrahydrofuran) will give complete recovery of fecal ^{14}C.

Feces Samples

The milled (and sieved) feces samples used in this experiment were the same feces samples employed in Experiments 2 and 6. They were SRF, BL-13-24, CT-4-24, CT-4-48, SMF, and 75-5-48.

Solvents

Benzene - ACS Reagent (Matheson, Coleman & Bell)

Ethyl Acetate - ACS Certified (Fisher Scientific Co.)

Acetonitrile - Reagent Grade (Matheson, Coleman & Bell)

Tetrahydrofuron - HPLC Grade (Fisher Scientific Co.)

Protocol

Placed 1 g aliquots of milled feces in 15 ml screw cap centrifuge tubes and extracted with organic solvents sequentially as follows:

A. Extracted with 10 ml benzene followed by extraction with 5 ml benzene.

B. Extracted residual solids with 10 ml ethyl acetate followed by extraction with 5 ml ethyl acetate.

C. Extracted residual solids with 10 ml acetonitrile followed by extraction with 5 ml acetonitrile.

D. Extracted residual solids with 10 ml tetrahydrofuran followed by extraction with 5 ml tetrahydrofuran.

For each extraction the tubes were shaken for 30 min, centrifuged for 10 min, and the supernatant solvent phases poured into a LSC vial. The extracts for each solvent were combined and evaporated under N_2 in a Mayer N-evaporator. Each residue was dissolved in 1 ml THF, bleached, if required, and counted in Biofluor.

Results

1) Ethyl acetate extracted most of the fecal ^{14}C in all samples. (See Table 9).

2) 96% of total dpm's was recovered in the total benzene and ethyl acetate extracts for all samples except the feces samples of treated but non-operated rat CT-4.

3) Acetonitrile extracted 15% of total dpm's in both feces samples from rat CT-4.

4) Tetrahydrofuran recovered little more than 1% of total dpm's except in rat CT-4 (CT-4-24, 4.1%; CT-4-48, 6.6%).

5) Two previously assayed samples, CT-4-48 and 75-5-48 showed lower recoveries in this experiment when compared to recoveries in earlier studies (See Table 5).

Discussion

Sequential extraction with the 4 organic solvents studied did not give total recoveries of ^{14}C in two treatment feces samples as high as those we obtained in earlier studies. This indicates that we might have recovered additional ^{14}C by digesting the residues with NaOH or Soluene.

It is quite interesting that 15% of the total dpm's in both non-operated rat samples (CT-4-24 and CT-4-48) was recovered in acetonitrile. This suggests that acetonitrile will extract fecal metabolites present in rat treatment feces samples.

Conclusions

The 4 organic solvents extracted most of the fecal ^{14}C, but % dose recoveries were lower for 2 samples than those obtained in earlier studies. This difference is under investigation.

Further work is planned to find the best series of solvents for sequential extraction and separation of ^{14}C - containing metabolic fractions of feces.

Table 1.9

Sequential Extraction of Six Feces Samples with Four Organic Solvents

Solvent (Sequential)	dpm/g					
	SRF	BL-13-24	CT-4-24	CT-4-48	SMF	75-5-48
1. Benzene	1042 (12.3)*	18685 (20.9)	5660 (12.2)	353 (21.3)	7863 (29.5)	10963 (10.0)
2. Ethyl Acetate	7228 (85.3)	66980 (74.9)	32314 (69.5)	932 (56.3)	17889 (67.2)	94283 (86.3)
3. Acetonitrile	161 (1.9)	3105 (3.5)	6614 (14.2)	260 (15.7)	624 (2.3)	3381 (3.1)
4. Tetrahydrofuran	42 (0.5)	655 (0.7)	1921 (4.1)	109 (6.6)	260 (1.0)	578 (0.5)
Total	8473	89425	46509	1654	26636	109205
	(100.0)	(100.0)	(100.0)	(99.9)	(100.0)	(99.9)

* % of total dpm's in each extract

1.10 SUMMARY

1. We have developed a procedure for preparing uniform fecal samples for LSC counting and study of fecal metabolites. The procedure requires milling of a rat or monkey feces sample with anhydrous Na_2SO_4 in a stainless steel ball mill. The fine off-white flours that result are passed through a fine wire sieve (18-8 stainless steel wire, 30 holes/inch). This sieving increases homogeneity by removing traces of unground material and dietary fiber.
2. Digestion of samples with NaOH and counting in dioxane-toluene-naphthalene with Cab-O-Sil (DTN-C) gives reproducible recoveries. This procedure was adequate for 0.25 g and 0.5 g samples but inadequate for 1.0 g samples.
3. In a study of 7 solvent/digestants we concluded that the best solv/dig were 1) Soluene, 2) NaOH and 3) Protosol. These three solv/dig extracted the most dpm from feces samples and showed the best reproducibility for 0.25 g aliquots.
4. Protosol and Soluene digests count just as efficiently in Biofluor as in DTN-C.
5. Ten replicate Soluene digests counted in Biofluor and 10 replicate NaOH digests counted in DTN-C showed mean % recoveries which matched well (NaOH, 40.6 ± 0.32 SE; Soluene, 41.3 ± 0.43 SE).
6. Recovery of ^{14}C by combustion assay was compared with results obtained by digestion in NaOH, Soluene, and perchloric acid-H_2O_2. The combustion assays gave recoveries that agreed well with values obtained by our digestion procedures.
7. When 1.0 g aliquots of 4 milled feces samples were extracted sequentially 3 times with THF and digested once with Soluene, the THF extraction recoveries matched the digestion methods for monkey feces. Recovery of ^{14}C in rat

feces samples was 10 to 20% lower than digestion assays.

8. Six feces samples were extracted sequentially with benzene, ethyl acetate, acetonitrile and tetrahydrofuran to see whether this procedure would give complete recovery of fecal ^{14}C. 96% of total dpm's were recovered in the total ethyl acetate and benzene extracts for all the samples except the non-operated rat CT-4. In this rat only 78 to 82% was extracted by these solvents, suggesting that the metabolites in this rat's feces are different from those in monkey 75-5♀ (bile-duct cannulated). Most of the undissolved fraction in rat CT-4 was soluble in acetonitrile.
9. Further work is planned to find the best series of solvents for sequential extraction and separation of fecal ^{14}C containing metabolites. This will be followed by purification of the metabolic fractions on HPLC and eventual structural studies using GC-MS and other analytical techniques such as IR and NMR if sufficient material can be generated.

1.11 REFERENCES

1. Mahin, D.T. and Lofberg, R.T. A Simplified Method of Sample Preparation for Determination of Tritium, Carbon-14, or Sulfur-35 in Blood or Tissue by Liquid Scintillation Counting. Anal. Biochem. 16: 500-509, 1966.
2. Morrison, B.J. and R.A. Franklin. Rapid, Hygienic Method for the Preparation of Fecal Samples for Liquid Scintillation Counting. Anal. Biochem. 85: 79-85, 1978.

1.12 Appendix

Procedure for Preparing Uniform Fecal Samples and Measuring Total Radioactivity by LSC

Milling Protocol

1. If feces are moist remove water by blotting. Then weigh the frozen feces sample.
2. Place the feces sample and a known amount (4 to 8 parts) of anh. Na_2SO_4 in a 0.5 gal. stainless steel ball mill. Add approximately 100 ¼" and 8 - 12 1" stainless steel balls. (The number of balls is increased or decreased in accordance with sample size).
3. Mill the sample for 30–60 min. (Time may be increased for very large samples).
4. Inspect the milled feces to see that there are no grossly discernible large unmilled particles.
5. Pass the milled feces sample through a fine wire sieve (18-8 stainless steel wire, 30 holes/in.). Significant amounts of unground material are reground and combined with the rest of the powder.
6. Store feces powder at -20°C.

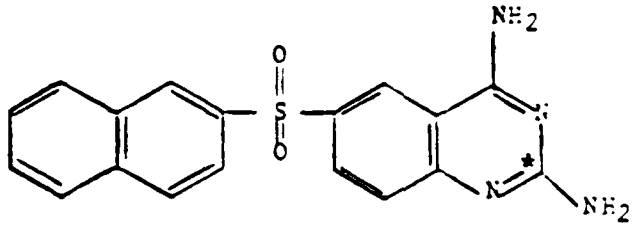
Digestion Protocol

1. Weigh milled feces samples (Approximately 250 mg) on an analytical balance and place in 20 ml LSC vials.
2. Add 1 ml of solvent/digestant (Soluene, NaOH (0.5 N) or Protosol).
3. Digest in oven at 68-70°C for 24-48 hr.
4. Bleach by adding 0.5 ml tertiary butyl hydrogen peroxide (TBHP) at 2-24 hr.
5. Add either 15 ml of 5% DTN-Cab-O-Sil (DTN-C) or 15 ml of Biofluor.
6. Cool and dark-adapt samples at least 2 hr at 4°C before counting.
7. Internally standardize with toluene ^{14}C, if desired.

SECTION 2. ADDITIONAL STUDIES ON METABOLISM OF WR-158,122 IN RATS AND MONKEYS

2.1 Introduction

Studies on the metabolic fate of WR-158,122 [2, 4-diamino-6-(2 naphthylsulfonyl)-quinazoline-2-^{14}C], the antifolate antimalarial compound, shown below



have been carried out in 3 rhesus monkeys, 10 bile-duct cannulated rats, 10 bile-duct ligated rats and 8 controls rats. Data on excretion and residual tissue distribution in rats and blood level data and excretion in monkeys are detailed in this report.

2.2 MATERIALS AND METHODS

Compounds. WR-158,122 [2,4-diamino-6-(2-naphthylsulfonyl)-quinazoline-2-^{14}C], empirical formula $C_{18}H_{14}SO_2N_4$, mol. wt. 350, was supplied by Walter Reed unlabeled as Bottle AY 65859. The ^{14}C-labeled preparation was synthesized by Research Triangle Institute and dated 1/8/79. Lot No. 2572-110, dated 8/20/79, has a specific activity of 69 µCi/mg or 24 mCi/mmole. The radiochemical purity in a number of TLC systems was > 98%.

Treatment Suspension. A typical treatment suspension was prepared as follows: 307.13 mg cold drug and 3.08 mg of ^{14}C labeled drug was ground intimately in a glass mortar with glass pestle with addition of small amounts of diluent (0.2% methylcellulose and 0.4% Tween 80 in distilled water) until a smooth suspension was achieved. The suspension was decanted into a tared round-bottom polycarbonate 200 ml centrifuge bottle containing glass beads and diluted to 124.08. It contained 2.50 mg WR-158,122/ml and 1.86 µCi/ml. The suspension was stored at 4°C. Assay of this suspension gave the following results:

4,136,000 dpm/ml
1654 dpm/µg
1.86 µCi/ml

Other preparations with approximately the same activity were prepared in the same manner.

Analytical Procedures:

Blood samples were analyzed for total ^{14}C content as described in Interim Report No. 2. Urine samples from rats or monkeys were counted in Biofluor® (New England Nuclear Corp.). Feces were assayed in a few instances by the total sample digestion procedure described in Interim Report No. 1. Because we wished to identify fecal metabolites as well as measure total ^{14}C

content, most samples were milled in a stainless steel jar with stainless steel balls according to the procedure developed in this laboratory and described in detail in Interim Report No. 4. In brief, the samples were ground in the ball mill for 30 min. with 4 to 8 parts of anhydrous Na_2SO_4. Aliquots of the resulting fine off-white powder (250 mg) were digested for 48 hrs. with 1 ml of Soluene® (Packard Instrument Co.) and after bleaching with 0.5 ml of t-butyl hydrogen peroxide were counted in Biofluor. All samples were initially cooled and dark adapted and were internally standardized to determine efficiency.

The carcasses and other tissues (either separately in some cases or as a unit in others) were counted as described previously. The bones do not dissolve in the alkaline digestion procedure employed. However, digestion with Soluene® of the dried finely powdered bones from some representative rats yielded counts equivalent only to 0.01% of the dose. Extraction of the bone powder with tetrahydrofuran recovered even smaller amounts of drug (as ^{14}C). Based on these findings no further work on bone ^{14}C has been done.

Animals. Rats. Sprague-Dawley-derived rats weighed 244 to 305 g (bile duct ligated), 260 to 400 g (bile duct cannulated) and 365 to 410 g (control rats) at the time of treatment. The bile duct ligated rats were prepared as described in Interim Report No. 1 and housed in metabolism cages. The bile duct cannulated rats prepared essentially as described in Interim Report No. 1, were restrained in a close fitting wire housing attached to the metal cover of a plastic cage. Food and water were supplied ad libitum four hours after dosing. Urine and feces were separated using a bottle equipped with a funnel and woven wire separator. Bile was collected in a

tared vial.

Bile, urine and feces were collected usually at 12, 24, and 48 h. At 48 h. or 72 h. the rats were necropsied using an anesthetic dose of Nembutal®. In a number of instances the tissues were assayed for ^{14}C in three sections: 1) tissues (liver, heart, lungs, spleen and kidneys as a pool); 2) gastrointestinal tract and contents; 3) carcass. However, these analyses have been reported as a single item, "carcass with all tissues", since with rare and usually unexplained exceptions these three tissue groups accounted for no more than about 2% of the dose.

Ten bile duct ligated rats were prepared and all are reported. Of the 20 bile duct cannulated that were prepared only 10 are reported. Four died prematurely; 3 exhibited unacceptably low recovery; two had large amounts of drug in the GI tract not characteristic of any of the other animals in the group; and one was eliminated because it had unaccountably large amounts of ^{14}C in the carcass.

Twelve control rats were given WR-158,122 but only 8 are reported. Three were deleted because of low recovery (50 to 60%) of the ^{14}C dose. The basis for these poor recoveries has not been established. One was eliminated because about half the dose was present in the carcass whereas the other carcass values were well below 1%.

Monkeys. Two unoperated rhesus monkeys (# 1449♀ and # 709♀) were administered WR-158,122 while in a primate chair. Both animals required supplemental feeding possibly due to the stress associated with the restraint.

The bile duct cannulated monkey (75-5♀) was acclimated to a primate chair for considerable periods prior to surgery. The cannulation was accomplished under Ketamine®-Surital® anesthesia following a 24 h fast. Blood chemistries

and CBC were done on a blood sample obtained at the time of the operation. After the operation the animal was given Flocillin® (1 ml BID) and intravenous fluids. When no bile flow occurred on day 4 surgery was repeated. Following a two week period during which the animal required considerable support in the form of bile, fluids and liquid diet given via nasal catheter or duodenal tube, the animal's bile flow became more consistent. On the twentieth day following the second operation, the animal was given a single oral dose of WR-158,122. During the post treatment period saline was slowly infused via the duodenal tube and the animal's bile was replaced with about equal amounts of beef bile. Blood samples were obtained from the left or right antecubital veins at 2, 4, 6, 8, 12, 24, 48 and 72 hours after treatment. Bile production and feces and urine excretion were excellent during this period.

A second dose of WR-158,122 was administered at 4 days. During the second post-operative period the animal also required supportive fluids and beef bile but bile, urine and feces production was good during this period.

2.3 Results

Bile Duct Ligated Rats. The excretion of single oral doses of labelled WR-158,122 in 10 bile duct ligated rats is shown in Table 1. Urinary excretion varied from 11 to 21% and was essentially complete at the end of 48 hours. Fecal excretion varied from 74 to 93 per cent of the dose. The high value for total recovery in BL-10 is probably due to an undetected error in measuring the treatment dose. As one would expect, the 4 rats sacrificed at 48 hours (Bl-15 to Bl-18) had more drug (1 to 11%) in their tissues at necropsy than the remaining animals killed after 72 hr (0.3 to 0.7%) (see Table 2), but most of the drug was cleared from the rat's body by 72 hr. Additional

comments on these data will occur later in the report.

Bile Duct Cannulated Rats. The excretion of single oral doses of labeled WR-158,122 in 10 bile duct cannulated rats is described in Table 3. Biliary excretion (as ^{14}C) varied rather widely from 2.5 to 23.6%. This variation may reflect the adequacy of biliary excretory processes but does not seem to depend on bile flow. By far the most active bile excretion occurred during the first 12 hours after treatment; the fraction of the total biliary excretion which occurred during this first period varied from 59 to 87%.

Urinary excretion of WR-158,122 (as ^{14}C) in bile duct cannulated rats accounted for from 0.8 to 4.2% of the dose, the major excretion via this route occuring usually during the first 12 hours.

Fecal excretion of WR-158,122 (as ^{14}C) in bile duct cannulated rats varied from 64 to 94% of the dose. Excretion via this route occurred principally during the first 24 hr period and was essentially complete by 48 hours, as indicated by the finding that less than 1% of the dose was recovered in the GI tract and contents at 48 hours when the rats were necropsied.

The data on recovery of ^{14}C in the carcass and tissues presented in Table 4 show that from 0.42 to 3.45% of the dose was still in the body at the end of 48 hours. As a rule the amount of drug (as ^{14}C) in the body at this time was 1% or less, clearance of drug from the body being essentially complete by 48 hours.

Control Rats. The parallel results obtained in 8 control rats are shown in Tables 5 and 6. The kinetics of excretion were essentially the same as observed in bile duct ligated and bile duct cannulated rats. Urinary excretion accounted for 0.8 to 2.9% of the ^{14}C administered and occurred primarily (68 to 91%) during the first 12 hours.

Fecal excretion (as ^{14}C) accounted for from 91 to 109% of the dose, the principal portion occurring during the first 24 hours.

The drug content (as ^{14}C) of the carcass at 48 hours never accounted for more than about 0.5% of the dose. This finding further supports the suggestion that in rats WR-158,122 is rapidly cleared from the body after single oral doses.

If we compare data in bile duct ligated, bile duct cannulated and control rats (Table 7) one is led to some interesting conclusions. Comparison of the data on urinary excretion of WR-158,122 in bile cannulated rats with the data in control (unoperated) rats suggests that about equal amounts of the drug undergo urinary excretion whether the bile duct is cannulated or not. If the duct is ligated, however, considerably more ^{14}C appears in the urine (16%). These findings suggest that at least 13 to 16% of the dose must be absorbed, some being excreted in the urine (1.6-2.3%), the rest in the bile. The assumption that the livers of these bile duct ligated and bile duct cannulated animals may not be functioning as well as in unoperated animals suggests that perhaps even more of the dose normally undergoes absorption and biliary excretion. The fact that the drug is cleared rapidly from the GI tract (data obtained but not shown) suggests that that portion of the drug undergoing biliary excretion is not reabsorbed. Perhaps the only metabolites excreted in the bile are conjugates too polar to be reabsorbed.

Monkeys

Although biliary cannulation was carried out in four monkeys, only one (75-59) survived for sufficient time to permit metabolism studies. No pertinent reasons have been discovered to account for this rather poor survival

record. In Table 8 blood chemistries on 75-59 at the time of initial surgery and at two periods thereafter are compared with normal values. The animal had high levels of LDH and alkaline phosphatase and slightly elevated SGOT even before surgery; values for other parameters were close to normal values.

Following a single oral dose of WR-158,122 (5 mg/kg), the highest blood or plasma level occurred at 2 hours; then the levels fell to almost insignificant values by 24 hours and were below the level of detection thereafter (Table 9). Because of the stress associated with collecting blood samples in this monkey, blood levels were not determined during the second treatment period.

Excretion of WR-158,122 (as ^{14}C) in bile, urine and feces following two single oral doses of WR-158,122 are detailed in Table 10. Recovery of drug (as ^{14}C) in bile was quite low, equal to less than 1% of the dose. About 3% was recovered in the urine and the remainder in the feces. Comments on these data appear in the discussion.

Two unoperated monkeys were used to evaluate the absorption and excretion of WR-158,122. The clinical chemistry findings (SMA 12/60) compared with results in a group of 20 healthy female rhesus monkeys studied by the same laboratory but housed in another building are shown in Table 11. Except for the elevated LDH values for which we have no explanation, these two female animals appeared to be relatively normal, as judged by these data.

The excretion of WR-158,122 (as ^{14}C) in these two female animals following single oral doses of 5 mg/kg are shown in Table 12. It is interesting that although total recovery of ^{14}C amounted to only 84 to 87%, from 14 to 18% of the ^{14}C appeared in the urine, the remainder in the feces.

2.4 Discussion

It is interesting to compare the data in this Interim Report with the results we obtained previously in Interim Report No. 1. In rats with bile duct ligation, the greatest difference appeared in fecal recovery (81.5% in current group versus 67.8% in the Interim Report No. 1 group) and higher total recovery (99.9% in Interim 5 versus 88.4% in Interim 1). Excretion in bile was slightly better in the early group of rats.

In the bile duct cannulated rats there was a greater disparity between the two groups of animals. Thus we recovered almost 30% of the dose of WR-158,122 in the bile in the Interim 1 group but only 11% in the current series. The simplest explanation for this discrepancy might be that the bile duct cannulae appeared to function better in the first group of rats. Comparison of the kinetics of biliary excretion in the two groups indicates another striking difference. In the first group of six rats (Interim 1) the peak excretion of ^{14}C in the bile occurred in the 12-24 hr. sample in 3 rats, in the 24-48 hr sample in 2 rats and in the 0-12 hr. sample in only 1 rat. In contrast, in the 10 rats included in this interim report all of the rats gave maximum values for biliary excretion in the 0-12 hr. period after which the excretion rates dropped rather rapidly in most instances. The basis for this difference has not been ascertained but one would reason that if the biliary cannulae are functioning well excretion via this route would be greater than in a group of rats with less well functioning biliary cannulae. Perhaps we need additional criteria on which to evaluate the adequacy of the cannulation procedure.

The monkey data suffers from its paucity as well as from the general comment that the data in this one bile duct cannulated monkey is probably

not very representative of data one should expect in healthy monkeys with adequate biliary cannulae. Thus one would expect that the total excretion of WR-158,122 (^{14}C) in bile and urine in monkeys with good bile duct cannulae should at least equal the urinary excretion of the drug in intact monkeys. If the drug is excreted in the bile as a non-reabsorbable metabolite then combined urine and biliary excretion in bile cannulated animals could very well exceed the urinary levels observed in intact animals. However, such has not been our experience to date and this suggests that the biliary cannulation technique needs improvement.

It might be worth commenting in this connection that urinary excretion of ^{14}C in control rats given single oral doses of WR-158,122-^{14}C was about one order of magnitude less than the corresponding figures in either bile duct ligated rats or bile duct cannulated rats (when in the latter one adds the biliary excretion to the urinary values). The best explanation of this would appear to be that the rat excretes WR-158,122 as metabolites (conjugates) in the bile that are unable to be reabsorbed from the gut.

In the unoperated monkey, urinary excretion levels are about 10 times as high as in the rat and this suggests either that the total metabolites excreted in the bile is considerably less in the monkey than in the rat or that the form in which the monkey excretes the compound in the bile is much more readily reabsorbed and excreted in the urine.

2.5 Summary

1. Blood levels, excretion and tissue distribution of WR-158,122 ^{14}C were studied in 10 bile duct ligated rats, 10 bile duct cannulated rats, 8 control rats and in 2 control and one bile duct cannulated monkeys.
2. In 72 hours bile duct ligated rats excreted about 16.3 ± 3.8% as ^{14}C in the urine and the remaining ^{14}C was recovered primarily in the feces. About 2% was recovered in the carcass and tissues at necropsy.
3. Bile duct cannulated rats excreted about 10.9 ± 6.1% in the bile, 2.3 ± 1.3% in the urine and the remainder in the feces. Only about 1.3 ± 1.0% was recovered in the carcass and tissues.
4. Control rats excreted the compound rather rapidly; 1.5 ± 0.8% of the ^{14}C was recovered in the urine, 0.3 ± 0.2 in the carcass and other tissues and the remainder in the feces.
5. Total average recovery (as ^{14}C) in the three groups of rats varied from 94 to 100% of the dose.
6. Two experiments were conducted in a single bile duct cannulated monkey. In the first the blood and plasma levels were highest at two hours after dosing. Plasma levels routinely exceeded levels in whole blood indicating little sequestration of WR-158,122 (as ^{14}C) in the red cells. Both levels were low at 24 hours and below detection at 48 hours.
7. In this bile duct cannulated monkey biliary excretion over a 96 h. period accounted for 0.6 to 0.9% of the dose (as ^{14}C), urinary excretion for 3.1% and 87 to 95% of the dose was recovered in the feces.
8. In two control monkeys urinary excretion over a 96 hour period amounted

to 14 and 18%; 70% of the dose (as ^{14}C) was recovered in the feces. Excretion was essentially complete after 96 hours.

9. In both rats and monkeys WR-158,122 appears to be rapidly but incompletely absorbed. Rats excreted 10% more in the bile but only 2-3% in the urine except in the case of bile duct ligated rats which excreted about 16% of the dose (as ^{14}C) in the urine.

Table 2.1

Excretion of WR-158,122 in Bile Duct Ligated Rats
Single Oral Dose of 10 mg/kg

Sample	Hours Post Dose	Percent Dose Recovered as ^{14}C									
		BL-9	BL-10	BL-11	BL-12	BL-13	BL-14	BL-15	BL-16	BL-17	BL-18
Urine (ml)	6	1.4 (10.0)	1.5 (7.2)	1.1 (10.0)	1.2 (9.9)	3.1 (11.3)	1.0 (10.2)	*NC	NC	NC	NC
	12	6.1 (10.3)	5.5 (13.0)	7.4 (11.2)	4.9 (10.7)	6.0 (9.9)	5.1 (6.7)	5.8 (8.5)	6.5 (16.5)	6.8 (14.0)	6.6 (13.0)
	24	3.9 (10.8)	6.2 (12.4)	10.1 (10.2)	6.1 (10.4)	7.0 (10.7)	1.5 (10.9)	5.3 (20.5)	2.4 (31.5)	5.6 (34.0)	4.8 (14.0)
	48	1.6 (27.5)	6.7 (41.0)	2.0 (13.5)	4.4 (23.0)	4.4 (51.0)	5.1 (33.5)	1.6 (35.5)	2.2 (35.5)	5.7 (30.0)	3.9 (30.5)
	72	0.23 (15.0)	0.83 (18.5)	0.42 (16.5)	0.59 (15.0)	0.45 (10.5)	0.41 (15.0)	**SAC	SAC	SAC	SAC
Total		13.2	20.7	21.0	17.2	21.0	13.1	12.7	11.1	18.1	15.3
Feces (g)	24	79.1 (3.0)	82.3 (3.8)	69.3 (5.1)	69.8 (3.3)	71.2 (4.9)	65.5 (4.7)	63.5 (3.3)	84.3 (8.0)	68.4 (6.6)	60.7 (1.9)
	48	5.2 (4.2)	10.8 (5.9)	4.6 (5.5)	11.0 (2.6)	6.4 (4.0)	14.5 (3.7)	14.4 (2.8)	2.1 (3.5)	12.0 (4.3)	17.2 (1.6)
	72	0.39 (4.8)	0.23 (4.4)	0.10 (3.6)	1.5 (5.1)	S.L.+	1.0 (2.7)	SAC	SAC	SAC	SAC
Total		84.7	93.3	74.0	82.3	77.6	81.0	77.9	86.4	80.4	77.9

*NC = No collection

**SAC = Sacrificed at 48 hrs.

+S.L. = Sample Lost

Table 2.2

Recovery of WR-158,122 in Bile Duct Ligated Rats
Single Oral Dose of 10 mg/kg

	Percent of Dose Recovered (as ^{14}C) in 48* or 72 Hours									
	BL-9	BL-10	BL-11	BL-12	BL-13	BL-14	*BL-15	*BL-16	*BL-17	*BL-18
Urine	13.2	20.7	21.0	17.2	21.0	13.1	12.7	11.1	18.1	15.3
Feces	84.7	93.3	74.0	82.3	77.6	81.0	77.9	86.4	80.4	77.9
Carcass with all tissues	0.36	0.53	0.35	0.53	0.46	0.72	0.93	2.4	2.6	10.9
Total	98.3	114.5	95.4	100.0	99.1	94.8	91.5	99.9	101.1	104.1

Table 2.3

Excretion of WR-158, 122 in Bile Duct Cannulated Rats

Single Oral Dose of 10 mg/kg

Sample	Hours Post Dose	Percent Dose Recovered as ^{14}C									
		BC-13	BC-14	BC-15	BC-17	BC-18	BC-21	BC-22	BC-23	BC-24	BC-26
Bile	12	16.3	7.4	8.6	3.4	2.1	9.1	10.4	10.1	4.7	6.3
(g)		(8.3)	(6.7)	(6.8)	(8.3)	(8.2)	(10.1)	(8.8)	(7.8)	(7.4)	(7.7)
	24	5.2	1.7	4.7	0.37	0.27	0.95	4.4	1.4	2.2	0.91
		(8.0)	(13.5)	(6.4)	(3.7)	(7.3)	(9.1)	(8.9)	(7.0)	(10.2)	(8.3)
	48	2.1	0.52	1.9	0.32	0.14	0.40	0.68	0.60	1.1	0.48
		(17.9)	(16.4)	(13.6)	(14.8)	(16.8)	(21.0)*	(21.2)*	(47.0)*	(27.9)*	(15.2)
Total		23.6	9.6	15.2	4.1	2.5	10.5	15.5	12.1	8.0	7.7
Urine	12	1.5	1.3	1.1	0.60	0.39	3.33	1.5	1.5	0.93	1.0
(ml)		(9.4)	(2.9)	(3.4)	(6.6)	(10.6)	(13.2)	(8.8)	(10.8)	(15.8)	(4.7)
	24	0.68	0.57	2.1	0.13	0.08	0.35	1.9	0.34	0.39	0.16
		(13.0)	(15.6)	(8.3)	(6.3)	(12.6)	(6.3)	(10.2)	(9.0)	(11.8)	(4.7)
	48	0.31	0.20	1.0	0.07	0.15	0.19	0.33	0.28	0.32	0.13
		(26.3)	(17.6)	(26.4)	(12.6)	(19.6)	(57.0)	(54.0)	(41.0)	(25.2)	(17.8)
Total		2.5	2.1	4.2	0.80	0.62	3.9	3.7	2.1	1.6	1.3
Feces	24	54.4	62.4	82.4	63.2	82.4	88.5	78.8	68.3	81.3	71.9
(g)		(3.0)	(6.5)	(2.2)	(3.1)	(8.8)	(17.6)	(6.6)	(4.3)	(7.3)	(2.1)
	48	10.0	4.8	12.0	15.6	1.4	1.7	3.6	7.1	3.9	20.4
		(10.5)	(14.3)	(9.0)	(6.1)	(5.2)	(22.7)	(8.5)	(6.4)	(7.2)	(7.9)
TOTAL		64.4	67.4	94.4	78.8	83.8	90.2	82.4	75.4	85.2	92.3

* Sample volume increased with water

Table 2.4

Recovery of WR-158,122 from Bile Duct Cannulated Rats
Single Oral Dose of 10 mg/kg

	Percent of Dose Recovered (as ^{14}C) in 48 Hours									
	BC-13	BC-14	BC-15	BC-17	BC-18	BC-21	BC-22	BC-23	BC-24	BC-26
Urine	2.5	2.1	4.2	0.80	0.62	3.9	3.7	2.1	1.6	1.3
Feces	64.4	67.2	94.4	78.8	83.8	90.2	82.4	75.4	85.2	92.3
Bile	23.6	9.6	15.2	4.1	2.5	10.5	15.5	12.1	8.0	7.7
Carcass with all tissues	1.37	0.56	3.45	1.03	2.35	0.76	0.42	0.71	1.94	0.66
Total	91.9	79.5	117.3	84.7	89.3	105.4	102.0	90.3	96.7	102.0

Table 2.5

Excretion of WR-158,122 in Control Rats
Single Oral Dose of 10 mg/kg

Sample	Hours Post Dose	Percent Dose Recovered as ^{14}C							
		CT-1	CT-2	CT-3	CT-4	CT-8	CT-9	CT-11	CT-12
Urine (ml)	12	1.7 (13.0)	1.2 (4.7)	2.0 (11.0)	1.3 (8.0)	0.74 (8.8)	1.3 (8.8)	0.60 (7.5)	0.68 (6.1)
	24	0.73 (14.5)	0.12 (8.0)	0.8 (10.8)	0.28 (13.0)	0.06 ((5.5)	0.38 (13.9)	0.07 (11.8)	0.11 (10.3)
	48	0.07 (31.0)	0.03 (14.5)	0.13 (24.0)	0.04 (18.0)	0.01 (7.1)	0.03 (19.4)	0.02 (32.0)	0.03 (7.2)
Total		2.5	1.4	2.9	1.6	0.81	1.7	0.69	0.82
Feces (g)	24	83.0 (7.3)	95.9 (11.1)	97.5 (11.1)	90.8 (11.4)	84.5 (13.1)	107.3 (11.3)	94.3 (11.2)	90.4 (9.3)
	48	16.1 7.1	2.8 (10.5)	2.8 (8.1)	6.5 (15.3)	6.1 (21.6)	1.3 (13.6)	1.4 (4.3)	5.0 (8.4)
Total		99.1	98.7	100.3	97.3	90.6	108.6	95.7	95.4

Table 2.6

Recovery WR-158,122 from Control Rats
Single Oral Dose of 10 mg/kg

	Percent of Dose Recovered (as ^{14}C) in 48 Hours							
	CT-1	CT-2	CT-3	CT-4	CT-8	CT-9	CT-11	CT-12
Urine	2.5	1.4	2.9	1.6	0.81	1.7	0.69	0.82
Feces	99.1	98.7	100.3	97.3	90.6	108.6	95.7	95.4
Carcass with all tissues	0.66	0.23	0.45	0.21	0.12	0.13	0.37	0.28
Total	102.3	100.3	103.7	99.1	91.5	110.4	96.8	96.5

Table 2.7

Recovery of WR-156,122 (as ^{14}C) in Bile Duct Ligated, Bile Duct Cannulated and Control Rats

	In Percent Dose								
	Bile-Duct Ligated			Bile-Duct Cannulated			Controls		
Sample	N	x	SD	N	x	SD	N	x	SD
Urine	10	16.34	3.78	10	2.28	1.28	8	1.55	0.81
Bile				10	10.66	6.14			
Feces	10	81.55	5.51	10	81.41	10.10	8	98.21	5.16
Carcass with all tissues	10	1.98	3.24	10	1.33	0.98	8	0.31	0.18
Total recovery	10	99.87	6.26	10	95.91	11.10	8	100.1	5.64

Table 2.8

SMA 12/60 Assays on a Bile Duct Cannulated Monkey Prior to Treatment With WR-158, 122 ^{14}C

(Rhesus 75-5 ♀)

	Sugar mg %	Uric Acid mg %	Chol mg %	SGOT mU/ml	LDH mU/ml	P mg %	Alk P mU/ml	Ca mg %	Direct Bili-rubin mg %	Total Bili-rubin mg %	Protein g %	Alb g %
Surgery 2-15-80	87	0.2	110	45	493	6.3	>350	10.8	0.2	0.5	8.1	4.0
2-25-80 7 days after 2nd surgery	79	0.7	80	40	355	4.5	>350	9.0	0.3	0.5	6.5	3.2
3-7-80 3 days before 1st treatment	85	0.3	80	40	330	5.9	>350	9.8	0.2	0.3	7.3	3.5
Normal* Values	89 ±3.5	0.61 ±0.05	170.2 ±6.0	30.4 ±2.2	271.2 ±24.6	3.56 ±0.21	166.3 ±17.5	9.78 ±0.16	0.12 ±0.01	0.26 ±0.02	8.01 ±0.13	3.77 ±0.11

*Based on assays of 20 rhesus female monkeys by same laboratory. (mean ± S.E.)

Table 2.9

Blood and Plasma Levels of WR-158,122 (as ^{14}C) in a Bile Duct Cannulated Monkey (75-5 ♀)

Single Oral Dose of 5 mg/kg

Hours Post Dose	First Treatment µg/g as ^{14}C		
	Blood	Plasma	Hct
2	0.33	0.52	41
4	0.26	0.40	40
6	0.17	N.S.*	N.S.*
8	0.13	0.20	40
12	0.05	0.09	40
24	0.02	0.05	42
49	<0.01	<0.01	38
72½	<0.01	<0.01	40

*No sample

Table 2.10

Cumulative Excretion of WR-158,122 (as ^{14}C) in Bile, Urine and Feces from a Bile Duct Cannulated Monkey (75-5 ♀)

Single Oral Doses of 5 mg/kg

Hours Post Dose	First Treatment Excretion in Percent of Dose						Combined Total
	Bile		Urine		Feces		
	Period	Total	Period	Total	Period	Total	
0-12	0.46	0.46	1.9	1.9			2.4
12-24	0.21	0.67	0.49	2.4	48.4	48.4	51.5
24-48	0.20	0.87	0.59	3.0	36.9	85.3	89.2
48-72	0.03	0.90	0.05	3.1	1.2	86.5	90.5
72-96	0.01	0.91	<0.01	3.1	0.10	86.6	90.6

Hours Post Dose	Second Treatment Excretion in Percent of Dose						Combined Total
	Bile		Urine		Feces		
	Period	Total	Period	Total	Period	Total	
0-12	0.26	0.26	1.5	1.5			1.8
12-24	0.21	0.47	1.1	2.6	35.8	35.8	38.9
24-48	0.13	0.60	0.37	3.0	57.9	93.7	97.3
48-72	0.01	0.61	0.11	3.1	1.0	94.7	98.4
72-96	0.01	0.62	0.02	3.1	0.09	94.8	98.5
96-120	<0.01	0.62	0.01	3.1	0.05	94.9	98.6

Table 2.11

SMA 12/60 Results on Two Control Monkeys Before Treatment

Monkey No.	Sugar mg %	Uric Acid mg %	Chol mg %	SGOT mU/ml	LDH mU/ml	P mg %	Alk. P mU/ml	Ca mg %	Direct Bili-rubin mg %	Total Bili-rubin mg %	Pro-tein g %	Alb g %
1449 ♀*	60	0.4	205	42	481	3.2	235	9.3	0.0	0.1	7.5	3.9
709 ♀**	51	0.3	170	44	446	2.5	151	9.6	0.0	0.1	7.3	3.9
Normal Values***	89 ±3.5	0.61 ±0.05	170.2 ±6.0	30.4 ±2.2	271.2 ±24.6	3.56 ±0.21	166.3 ±17.5	9.78 ±0.16	0.12 ±0.01	0.26 ±0.02	8.01 ±0.13	3.77 ±0.11

* assayed 2 days before treatment

** assayed 15 days before treatment

*** based on assays of 20 rhesus female monkeys by same laboratory (mean ± S.E.)

Table 2.12

Cumulative Excretion of WR-158,122 in Urine and Feces of Two Control Monkeys

Single Oral Doses of 5 mg/kg

Rhesus 1449 ♀					
Hours Post Dose	Urine*		Feces		Combined Total
	Period	Total	Period	Total	
0-12	2.6	2.6	0.02	0.02	2.6
12-24	7.8	10.4	0.17	0.19	10.6
24-48	2.9	13.3	51.9	52.1	65.4
48-72	0.59	13.9	11.0	63.1	77.0
72-96	0.15	14.1	6.9	70.0	84.1

Rhesus 709 ♀					
Hours Post Dose	Urine		Feces		Combined Total
	Period	Total	Period	Total	
0-12	6.0	6.0	N.S.**		6.0
12-24	8.5	14.5	0.13	0.13	14.6
24-48	2.2	16.7	0.02	0.15	16.9
48-72	0.71	17.4	58.3	58.5	75.9
72-96	0.24	17.6	10.5	69.0	86.6
96-120	0.05	17.7	0.59	69.6	87.3

* 24 and 48 hr. samples contaminated with menses

** N.S. = No Sample

APPENDIX

Table of Contents
Appendix

Table of Tables
Appendix

Table of Figures

ABSTRACT

A New Procedure for Preparing Uniform Fecal Samples for LSC. Carl C. Smith and G.F. Wolfe*, Dept. of Environmental Health, University of Cincinnati, College of Medicine, Cincinnati, Ohio 45267

We have devised a new procedure for handling radioactive (or regular) feces samples which circumvents many of the problems inherent in sampling feces. The entire sample is ground with 4 to 8 pts. of anh. Na_2SO_4 for 30 to 60 minutes in a stainless steel ball mill with stainless steel ball bearings. The resulting fine, off-white powder is passed through a fine wire sieve (18-8 stainless steel wire, 31 holes/in) to remove traces of unground material, principally fiber. Highly precise and reproducible values (usually with S.D. of 2 to 4%) were obtained using any of several counting procedures including combustion, Soluene^R/Biofluor^R, alkaline gels or perchloric acid. In addition to increased LSC efficiency (70-85% for ^{14}C), the preparation is an ideal starting material for characterizing fecal components and metabolities. No apparent changes occur during the procedure except dilution with the neutral salt. Samples can be extracted with organic solvents conveniently either in the batch mode in centrifuge tubes or chromatographically in columns or in a Soxlet apparatus. The application of these procedures in the characterization of the fecal metabolites of WR-158,122-^{14}C, an experimental antimalarial drug, will be illustrated. (Supported in part by Army Contract DAMD17-79-C-9106.

Presented at the ASPET meeting - August 17-21 in Rochester, Minn. (1980)

ABSTRACT

A New Approach to the Extraction of Polar Metabolites from Body Fluids. Carl C. Smith and G.F. Wolfe. Department of Environmental Health, University of Cincinnati, College of Medicine, Cincinnati, Ohio 45267.

Purpose of this study was to develop a new procedure for extracting polar metabolites of the antimalarial drug, WR-158,122 from body fluids such as urine or bile. For this purpose one needs a method which can utilize water miscible solvents such as tetrahydrofuran (THF), acetonitrile (ACN) or propanol (PRO). Saturating urine or bile with KBr (0.5 g/ml)permits one to extract from 85 to 98% of the total radioactivity of treatment urine or bile using 5 ml of PRO, dimethylformamide, pyridine or MeOH. All the ^{14}C in control urine spiked with parent drug is completely extracted by the above solvents, as well as by ethylene dichloride-2-ethylhexanol EDC-2EH, (8:2). EDC-2EH extracts only 3 to 4% of the total ^{14}C from rat or monkey treatment bile and 14 to 24% of the total ^{14}C from rat or monkey treatment urine. ACE, THF and PRO, in that order, extract increasing quantities of ^{14}C from rat or monkey treatment bile and urine. The exact quantities depend on type of animal (bile duct-ligated, bile duct-cannulated or unoperated control) which was used for the metabolism study. The solvents used are well adapted to subsequent HPLC studies and the procedure can be adapted to large samples if desired. (Supported in part by Army Contract DAMD17-79-C-9106).
(To be presented at the SOT meetings March 1-5, 1981 at San Diego, California)

Metabolism Studies on WR-158,122 in Bile Duct Cannulated Rats and Monkeys

DAMD 17-79-C-9106

Preliminary Report

February 16, 1981

Development of HPLC Procedures for the Isolation/ Identification of WR-158,122 Metabolites

M. Wilson Tabor, Ph.D.
Acting Principal Investigator
(7/1/80 - 6/30/81)

with the assistance of:

Geraldine F. Wolfe, M.S.

Department of Environmental Health and
Department of Laboratory Animal Medicine
University of Cincinnati College of Medicine
Cincinnati, Ohio 45267

PRELIMINARY REPORT
DEVELOPMENT OF HPLC PROCEDURES FOR THE ISOLATION/IDENTIFICATION
OF WR-158,122 METABOLITIES

INTRODUCTION

In interim report 4 (1980) we described the ^{14}C distribution data on the sequential extraction of 24 hour milled feces from bile duct ligated (BiDuLi or BL), bile duct cannulated (BiDuCa or BC), and control non-operated (CT) rats that had been treated with WR-158,122. The extraction procedure utilized a series of four solvents (benzene, ethylacetate,acetonitrile and tetrahydrofuran) of varying solubility parameters which sequentially extract nonpolar to polar compounds. In this preliminary report we are presenting our initial findings in the development of methods for the separation of these extracts to ultimately isolate and identify the major fecal metabolities of WR-158,122.

The general approach to compound/metabolite separation consists of:

1. Optimization of the feces sequential extraction procedure;
2. Preliminary examination of the fecal extracts by silica gel thin-layer chromatography (TLC) to obtain a general idea of the numbers and polarity of compounds in each extract;
3. and development of a silica gel high performance liquid chromatography method for separation of the samples for compound characterization and identification.

MATERIALS

Feces Samples

Rat feces (24 hours) from BiDuCa and Ct animals treated with 10 mg/kg oral doses of WR-158,122-^{14}C were milled and sieved as described in interim report 4. Specific animal samples are referenced for each experiment that follows.

Liquid Scintillation Counting(LSC)

Feces and solvent extracts were processed for LSC as described in interim report 4.

EXPERIMENT I

A STUDY OF THE EFFECT OF EXTRACT SHAKING TIME ON RECOVERIES IN SEQUENTIAL EXTRACTION SOLVENTS

INTRODUCTION

A preliminary sequential solvent extraction procedure has been developed for feces and was reported in interim report 4. The procedure involved extraction of the sample for 30 min with 10 ml of the appropriate solvent, and, following centrifugation and extract removal, a second 30 min extraction of the sample was performed using 5 ml of the same solvent. This time-consuming procedure was used for each of four solvents. We decided to optimize the extraction procedure to see if the extraction time could be shortened.

Purpose

To examine the length of fecal extraction time with regard to recovery of ^{14}C material.

Feces Samples (milled and sieved)

Six-one gram aliquots of BiDuCa-18-24 feces sample were used in this experiment. Superscripts refer to sample number.

Solvents

Benzene - ACS Reagent (Matheson, Coleman & Bell)

Ethyl Acetate - ACS Certified (Fisher Scientific Co.)

Acetonitrile- Reagent Grade (Matheson, Coleman & Bell)

Tetrahydrofuran - HPLC Grade (Fisher Scientific Co.)

Protocal

One gram aliquots of milled feces were placed in 10 ml screw-cap centrifuge tubes and were extracted as follows:

Part I - BC 18[5] BC 18[6] *	Part II-BC 18[7] BC 18[8]	Part III-BC 18[9] BC 18[10]
5 mins. of shaking time for each 5 ml of extract	10 mins. of shaking time for each 5 ml of extract	20 mins of shaking time for each 5 ml of extract

All samples were subjected to 3 x 5 ml extractions with these solvents in this sequence:

A. Benzene
B. Ethyl Acetate
C. Acetonitrile
D. Tetrahydrofuran

Pooled 15 ml of extract for each sample in LSC vials
Evaporated the extract in Meyer N-evaporator.
Dissolved residue in 1 ml of THF.

The residues of all extracts of BC-18[5], BC-18[7] and BC-18[10] were saved for HPLC analysis.

The residues from all extracts of BC-18[8] and BC-18[9] were processed for LSC and counted.

The BC-18[6] sample was lost during the extraction procedure.

Results

The LSC results of this experiment are summarized as follows:

	dpm/g of milled feces		
	BC 18[8] - 10 mins.	BC 18[9] - 20 mins.	%increased for 20 mins.
Benzene	8099	8287	2.3
Ethyl Acetate	49393	52087	5.5
ACN	581	568	2.2 *
THF	154	156	1.3

* a decrease

Discussion

The results of this experiment suggest a slight increase in recovery of ^{14}C with a 20 min extraction versus a 10 min extraction. The 5 min sample was not counted but a preliminary comparison of HPLC profiles suggest less recovery of

material in the 5 min extraction than in the 10 min extraction. HPLC methods are presented in a subsequent experiment.

Conclusion

We conclude that a 20 min extraction time should be given in the extraction procedures with milled feces.

EXPERIMENT 2

A STUDY OF THE EXTRACTING EFFECTIVENESS OF EACH SOLVENT ALIQUOT IN THE SEQUENTIAL EXTRACTION OF FECES SAMPLES

Introduction

A sequential solvent extraction procedure for feces has been developed and was reported in interim report 4. The solvent series -- benzene, ethylacetate, acetonitrile,tetrahydrofuran -- was used on feces as a 10 ml extraction followed by a 5 ml extraction for each solvent. We subsequently found that more consistent results were obtained if three 5 ml extractions were applied for each solvent. In this experiment we decided to examine the possibility of eliminating one of the 5 ml extractions for each solvent to conserve time and solvent for each extraction procedure.

Purpose

To determine the ^{14}C distribution and recovery in each extraction aliquot for each sequential solvent used in extracting rat feces.

Feces Samples

BC-18-24- The 24 hour milled and sieved feces sample from bile duct cannulated rat # 18 treated with WR-158,122^{14}C (10 mg/kg single oral dose) was used for this experiment.

Ct-3-24 - The 24 hr milled and sieved feces sample from control (non-operated) rat # 3 treated with WR-158,122^{14}C (10 mg/kg single oral dose) was used for this experiment.

Duplicates are indicated by superscripts 11 and 12.

Solvents

The solvents used for extraction were as described in experiment #1.

Protocol

1) Weigh 1 g of milled feces sample on analytical balance and place in a 10 ml screw-capped graduated centifuge tube. (4 feces samples)
2) Add 5 ml aliquots of the stipulated solvent to each tube for extraction. The solvent sequence is Benzene, Ethyl Acetate, Acetonitrile, Tetrahydrofuran.
3) Shake 20 minutes at high speed for each extraction.
4) Clarify by centrifugation for 3-4 minutes.
5) Transfer the extract to a pre-coded vial, indicating solvent and the extract number and prepare for counting.
6) Repeat steps 2-5 two additional times for each solvent.
7) 1.0 ml of the organic solvent is counted in 15 ml of Biofluor® scintillation medium, adapted at least 2 hours in LSC Counter.
8) After initial counting the sample is internally standardized with toluene ^{14}C.

Results

The results are summarized, indicating the dpm/g recovered for each extract for each solvent in Table 1. The total dpm recovered in the three extracts and the % of total dpm's recovered in each extract are also shown.

Conclusions

1) The second and third extractions with sequential solvents are requisite to good recovery with each solvent employed.
2) Some conclusions are clear-cut as to extraction differences between the two types of rat feces--bile duct cannulated vs. control (non-operated) rat feces as follows:

a. Control (non-operated) rat feces show slightly more uptake of dpm's in all three extracts of benzene.

b. Control rat feces have much greater uptake of dpm's in all acetonitrile extracts.

c. Control rat feces have much greater uptake of dpm's in all tetrahydrofuran extracts.

d. The three ethyl acetate extracts show quite a different uptake of dpm's in the two types of feces. The bile duct cannulated rat feces recover more dpm's in total ethyl acetate extract.

Table 1

Sequential Extraction of Milled Feces Samples
Recovery in Extraction Aliquots

Solvent (Sequential)	Extraction Number (5ml)	dpm/g BC 18[11]	dpm/g BC 18[12]	dpm/g CT 3[11]	dpm/g CT 3[12]
Benzene	1	3638 (46.9)+	3624 (47.0)	3727 (43.0)	3741 (40.4)
	2	2260 (29.1)	2429 (31.5)	2847 (32.9)	*3189 (34.5)
	3	1852 (23.9)	1665 (21.5)	2086 (24.1)	2322 (25.1)
	Total	7750 (99.9)	7718 (100.0)	8660 (100.0)	9252 (100.0)
Ethyl Acetate	1	45750 (87.0)	46250 (87.2)	29,940 (73.4)	30,415 (72.1)
	2	5880 (11.2)	5841 (11.0)	8070 (19.8)	8875 (21.0)
	3	970 (1.8)	921 (1.7)	2763 (6.8)	2891 (6.9)
	Total	52600 (100.0)	53012 (99.9)	40773 (100.0)	42,181 (100.0)
Acetonitrile	1	479 (63.5)	402 (61.9)	2580 (57.0)	2572 (56.0)
	2	200 (26.5)	170 (26.2)	1370 (30.3)	1415 (30.8)
	3	75 (10.0)	77 (11.9)	575 (12.7)	602 (13.1)
	Total	754 (100.0)	649 (99.9)	4525 (100.0)	4589 (99.9)
Tetrahydrofuran	1	130 (71.4)	113 (67.3)	1019 (67.2)	1078 (68.3)
	2	35 (19.2)	40 (23.8)	307 (20.3)	300 (19.0)
	3	17 (9.3)	15 (8.9)	190 (12.5)	200 (12.7)
	Total	182 (99.9)	168 (100.0)	1516 (100.0)	1578 (100.0)

*Leaked slightly
+ o/o of total dpm's for each solvent

EXPERIMENT 3

A PRELIMINARY TLC EXAMINATION OF FECES EXTRACTS

Introduction

In order to develop an adequate separation method by HPLC, samples are generally examined via TLC to obtain an indication of the type of chromatography sorbent to be used and the separation of the sample achieved by a particular solvent system. Furthermore the complexity of the sample can usually be ascertained by the distribution of compounds observed on TLC. Advantages of TLC as a preliminary screening method to HPLC include the small amount of time required to examine numerous samples and the relative small cost per sample examined. With these factors in mind, we decided to examine the methylene chloride extracts of feces from BiDu Ca and CT rats. Methylene chloride was used as a single extracting solvent because of its intermediate polarity and general extracting capabilities, particularly when only one solvent is used.

Purpose

To examine the extracts of feces from BiDu Ca and CT rats by TLC to aid in the development of an HPLC separation procedure.

Feces Samples

The feces samples used in this experiment were the same as those used in experiment 2. Duplicate samples are indicated by superscripts.

Solvents

Methylene Chloride - HPLC Grade (Burdick & Jackson)

Ethyl Acetate - ACS Certified (Fisher Scientific Co.)

Acetic Acid (Glacial) - ACS Reagent (Fisher Scientific Co.)

TLC

Thin-layer chromatography (TLC) was performed by standard techniques utilizing silica gel 60-F254 plates obtained from Merck via Fisher Scientific Co.

Ten to one hundred microliter portions of the feces extracts were spotted on the TLC plates at a distance of 2.0 cm from the bottom of the plate. Each plate was also spotted with 10 µl of a 2.5 ng/µl solution of WR-158,122. The TLC plates were developed in an ethylacetate:acetic acid (9:1 by volume) solvent system. Following chromatography, results were recorded for the visualization of spots in both 254 nm and 366 nm UV light. One centimeter scrapes were made of each chromatogram sample, beginning at 0.5 cm below the origin. The scrapings then were counted for ^{14}C in TSS #2 scintillator containing 0.1 mg DiMePoPoP and 4 mg PPO per ml in toluene.

Protocol

1. One gram aliquots of milled feces samples were placed in 10 ml screw cap graduated centrifuge tubes. Samples BC18^3, BC18^4, CT3^3 and CT3^4 were used.
2. The samples were each extracted for 30 min. by vigorous shaking with 5 ml of methylene chloride.
3. Following clarification by centrifugation for 5 min., step 2 was repeated twice.
4. The three methylene chloride extracts were pooled in an LSC vial and evaporated under N_2 in a Meyer N-evaporator.
5. Each residue was redissolved in 1.0 ml of tetrahydrofuran (THF) and divided as follows:

BC 18^3		BC 18^4	CT 3^3		CT 3^4
THF		THF	THF		THF
↓	↓	↓	↓	↓	↓
0.25 ml	0.75 ml	1.0 ml	0.25 ml	0.75 ml	1.0 ml
(TLC)	(LSC)	(HPLC)	(TLC)	(LSC)	(HPLC)

6. Samples were counted as described in interim report 4 and TLC was performed as described. BC 18^4 and CT 3^4 were saved for HPLC analysis.

Results

A. LSC Study

The LSC results on the methylene chloride extracts of feces are shown below. Recovery was ascertained by comparing the methylene chloride extract LSC data to soluene digests as shown.

	dpm/g milled feces	
	BC 18^3	CT 3^3
	53,378	41,016
	% dose as ^{14}C	
Methylene Chloride Extraction	66.7	53.6
Soluene Digestion	82.4	97.5

It is evident that methylene chloride extracts 1 g of milled feces with no more than 50-70% of total recovery of dose as ^{14}C.

B. TLC Study

For the methylene chloride extract of BC 18^3, parent compound (R_f = 0.16) and compound(s) at the origin were seen on visualization at 254 nm. A series of spots were seen in the R_f range of 0.7-0.9 at 366 nm.

For the methylene chloride extract of CT 3^3, parent compound (R_f = 0.16) and a series of discrete compounds (R_f = 0.1, 0.25, 0.4) were seen on visualization at 254 nm. The same series of spots seen with BC 18^3 (R_f = 0.7-0.9) were seen for this sample at 366 nm.

The results of LSC counting of the TLC scrapings of the methylene chloride extracts are:

BC 18[3]			CT 3[3]		
Strip	CPM	% of Total Count	Strip	CPM	% of Total Count
1 cm*	28	1.6	1 cm*	15	0.9
2 cm	15	0.9	2 cm	15	0.9
3 cm	98	5.7	3 cm	552	36.2
4 cm	1554	90.7	4 cm	927	60.9
5 cm	10	0.6	5 cm	7	0.5
6 cm	3	0.2	6 cm	3	0.2
7 cm	6	0.4	7 cm	4	0.3
Totals	1714	100.1	Totals	1523	99.9
15 cm	5	-	15 cm	5	-

*(the 1 cm strip includes both sides of the origin)

The counts in cm #1 probably are due to metabolites of WR-158,122-^{14}C in both BC 18[3] and CT 3[3] samples. The series of discrete compounds at R_f 0.1 seen at 254 nm for CT 3[3] are probably the metabolites that counted in cm #3. It is noted that only a small amount of the same metabolites are seen in BC 18[3] (LSC data). Further, the compounds visualized at 366 nm in both samples counted at values slightly above background as indicated for cm #15.

Discussion

Chromatography using a silica gel sorbent allows for the separation of polar and nonpolar substances in a mixture. In this system, the least polar compounds exhibit the largest R_f values, i.e. moving closest to the solvent front. The more polar substances have smaller R_f values and may even remain at the origin (R_f = 0).

The results of this preliminary TLC experiment suggest that WR-158,122-^{14}C is metabolized to compounds of higher polarity that are excreted in the feces. The majority of the radioactivity, other than the parent compound, was found at the origin or between the origin and the parent compound.

The low R_f value observed for the majority of the metabolites suggests the solvent system was not of sufficient polarity to obtain optimum chromatography. Although other solvent systems were tried, no extensive investigation was conducted to optimize the TLC system. The objective of the experiment was to examine the suitability of silica gel for further use as a HPLC chromatography sorbent for the separation of WR-158,122-^{14}C and its metabolites. The results suggest that silica gel HPLC would be an appropriate method for the desired separation. Also, the TLC method appears adequate for screening extracts as to the number and polarity of compounds that may present in a particular extract. This information also will aid in the further development of HPLC methods.

EXPERIMENT 4

PRELIMINARY DEVELOPMENT OF HPLC METHOD FOR THE SEPARATION OF WR-158,122-^{14}C AND ITS METABOLITES FROM FECES

Introduction

Development of a HPLC method for the separation of a mixture involves several steps. First an appropriate chromatographic sorbent has to be experimentally determined. The results of experiment 3 suggest silica gel to be a suitable sorbent. The next several steps of method development focus to determining an appropriate solvent system to achieve the separation. Achieving this, suitable standards are run to determine the limits of detection and separation for the system. The experimental samples are then run to examine them for similarities and differences. Finally, the system is scaled to the preparative scale to isolate sufficient material for compound identification. In the experiment described below, we have developed a preliminary HPLC method that is sufficient to examine similarities and differences in samples.

Purpose

To develop a HPLC method for the separation of WR-158,122-^{14}C and its metabolites that is suitable for the examination of similarities and differences in feces extracts from BiDuCa and CT rats.

Feces Samples

The feces samples used in this experiment were the same as those used in experiment 2. Duplicate samples are indicated by superscripts.

Extractions

Duplicate feces samples (BC 18^2, BC 18^1, CT 3^1 and CT 3^2) were extracted as described in experiment 1. Sample extracts were processed according to the procedure described in experiment 3. BC 18^2 and CT 3^1 were used for TLC and LSC. BC 18^1 and CT 3^2 were used for HPLC.

TLC

TLC was performed an BC 18^2 and CT 3^1 according to the method described in experiment 3. The resulting chromatograms were only examined visually at 254 nm and 366 nm. LSC of the chromatograms was not done.

Standard WR-158,122 Solutions

A stock solution (250 ng/µl) was made for WR-158,122 in tetrahydrofuran. Serial dilutions of 1 to 10 were made using tetrahydrofuran resulting in solutions containing 25 ng/µl and 2.5 ng/µl.

HPLC

High performance liquid chromatography (HPLC) separations were performed on a Waters Associates Model ALC/GPC 204 equipped with two 6000A pumps, a U6K injector, a solvent programmer and a 254 nm absorbance detector. The instrument was fitted with a Waters Radial Compression Module (RCM) unit containing an 8 mm by 10 cm column packed with 10 µm silica particles.

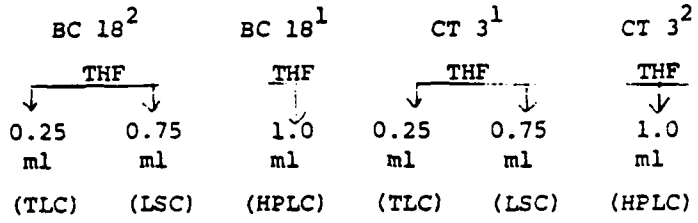
HPLC solvents were obtained as distilled-in-glass solvents from Burdick and Jackson laboratories. All HPLC solvents were degassed immediately prior to use by 15 min of sonication while under a partial vacuum.

Samples and standards for HPLC were injected as 2.5 µg to 100 mg per ml solutions in tetrahydrofuran (THF). The column was flushed with methylene chloride at 2.0 ml per min for 5 min. A 10 min linear gradient from 100% methylene chloride to 100% methanol was run, and then the column was eluted with methanol for an additional 10 minutes.

Protocol

1. Samples BC 18^1, BC 18^2, CT 3^1 and CT 3^2 were sequentially extracted with benzene, ethylacetate, acetonitrile according to procedure.

2. Following evaporation of the combined extracts, each residue was dissolved in 1.0 ml of tetrahydrofuran (THF), then divided as follows:



3. Aliquots of BC 18^2 and CT 3^1 were chromatographed on silica gel TLC, and the remaining portion was processed for LSC.

4. Appropriate HPLC separation parameters (solvents, flow etc.) were determined as described in the results.

5. BC 18^1 and CT 3^2 were examined by HPLC.

Results

A. LSC of Extracts

Results of LSC Study -

As in Experiment 7 (Interim Report No. 4) we found that ethyl acetate extracted most of the fecal ^{14}C in the sequential extraction. Ninety one to 98% of total dpm's were recovered in total benzene and ethyl acetate extracts. Acetonitrile extracted 6.4% of total dpm's in rat CT 3, whereas tetrahydrofuran extracted 2.1% of total dpm's. In contrast, dpm recoveries from BC 18^3 for these two solvents were less than 1% each. The LSC results are summarized as follows:

	dpm/g of milled feces (% dpm's)	
	BC 18[2]	CT 3[1]
Benzene	7406* (12.3)	9037 (17.9)
Ethyl Acetate	52147 (86.9)	37132 (73.6)
Acetonitrile	405 (0.67)	3239 (6.4)
Tetrahydrofuran	71.3 (0.12)	1077 (2.1)
Totals	60,029 (99.9)	50,485 (100.0)

*slight loss in extraction

B. TLC Results on Sequential Extraction Samples

The TLC results for each extract are summarized as follows:

Extract	R_f of Compounds Visualized			
	BC 18[2]		CT 3[1]	
	254 nm	366 nm	254 nm	366 nm
Benzene	0.0 0.16*	Series of three from 0.7-0.9	0.0 0.16	Series of three from 0.7-0.9
Ethyl Acetate	0.16	0.8	0.16	Many at 0.65-0.95
Acetonitrile	-	0.8	0.0	-
Tetrahydrofuran	-	0.8	0.0	-

*Parent compound

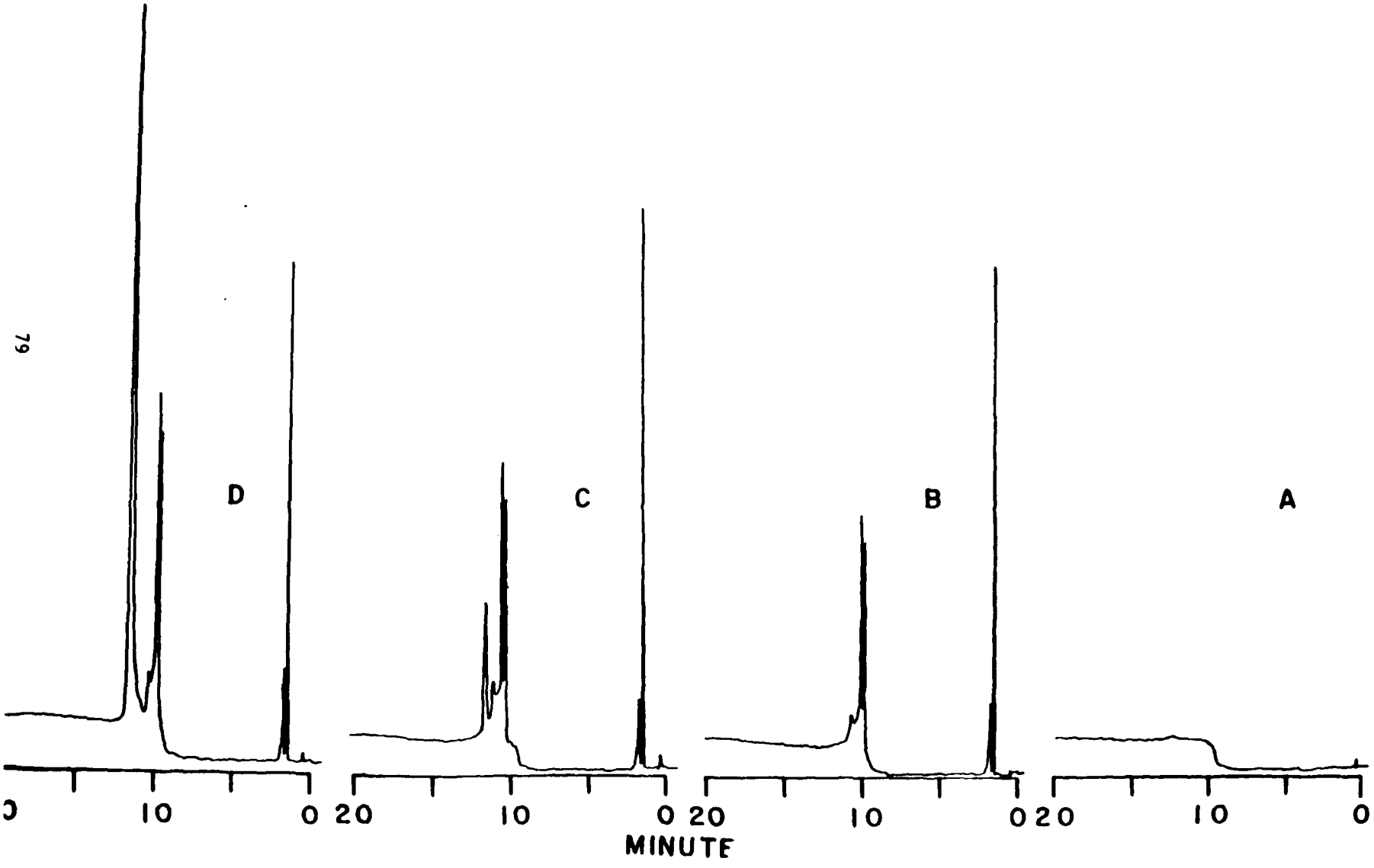
C. Development of the HPLC Separation

In a series of preliminary experiments, 25 µl of the standard solution of WR-158,122 (250 ng/µl) was injected into the HPLC, using a variety of solvent conditions. The compound did not elute when using 100% Hexane nor with 100% Methylene Chloride. With 100% Methanol, the compound eluted at the void volume of the column ($k^1 = 1.0$). Therefore, a HPLC procedure employing a linear gradient from 100% methylene chloride to 100% methanol was utilized. Twenty-five microliters of the standard solution was injected into the HPLC using 100% methylene chloride as the solvent. Following 5 min of flushing the column (5 column volumes), a 10 min linear gradient from 100% methylene chloride to 100% methanol was initiated. Following the gradient, the column was flushed an additional 5 min using 100% methanol. The results of this experiment and a series of control experiments are shown in Figure 1 and are summarized as follows.

The tetrahydrofuran sample solvent was found to contain several 254 nm absorbing compounds (Figure 1B). The parent compound was found to elute at 11.1 min as shown in Figures 1C and 1D. It appeared that this gradient method would suffice for a preliminary examination of feces extracts since the parent compound was resolved from the sample solvent contaminants and metabolites, being more polar than the parent, would be expected to elute near or after the parent compound.

Figure 1. HPLC Control Experiments for the Separation of WR-158,122.

Samples were injected at t=0; while the column was flushed at 2.0 ml/min with 100% methylene chloride; at t=5 min, a linear 10 min gradient from 100% methylene chloride to 100% methanol was initiated; at t=15 min, the column was flushed with 100% methanol for an additional 5 min. Chromatogram A=HPLC solvent blank; chromatogram B=25 µl of a tetrahydrofuran solution (2.5 ng/µl) of WR-158,122; chromatogram D=25 µl of a tetrahydrofuran solution (25.0 ng/µl) of WR-158,122.



D. HPLC Examination of the Extracts of BC 18[1] and CT 3[2] Feces

1. Benzene Extracts

The HPLC profiles of 25 µl of the tetrahydrofuran solutions of the benzene extracts of CT 3[2] and BC 18[1] are shown in Figure 2A and 2B, respectively. It appears that BC 18[1] has more metabolite(s) (t=14 min) than CT 3[2], whereas CT 3[2] appears to show the presence of parent. The early eluting peaks (t=1.5-3 min) are probably the fast moving material observed on TLC.

2. Ethyl Acetate Extracts

The HPLC profiles of 25 µl of the tetrahydrofuran solutions of the ethyl-acetate extracts of CT 3[2] and BC 18[1] are shown in Figure 3A and 3B, respectively. The majority of the parent compound appears in this extract for both samples. Note the absence of metabolites in the chromatogram which is in agreement with the TLC results.

3. Acetonitrile Extracts

The HPLC profiles of 25 µl of the tetrahydrofuran solutions of the acetonitrile ~~acetate~~ extracts of CT 3[2] and BC 18[1] are shown in Figure 4A and 4B, respectively. The BC 18[1] extract appears to contain little, if any, parent or metabolites, whereas the CT 3[2] appears to contain a trace of a metabolite. These results are consistent with the TLC observations.

Figure 2. HPLC Profiles of the Benzene

Extracts of feces. Chromatogram A=CT 3^2; chromatogram B=BC 18^1. Samples were run as described in Figure 1.

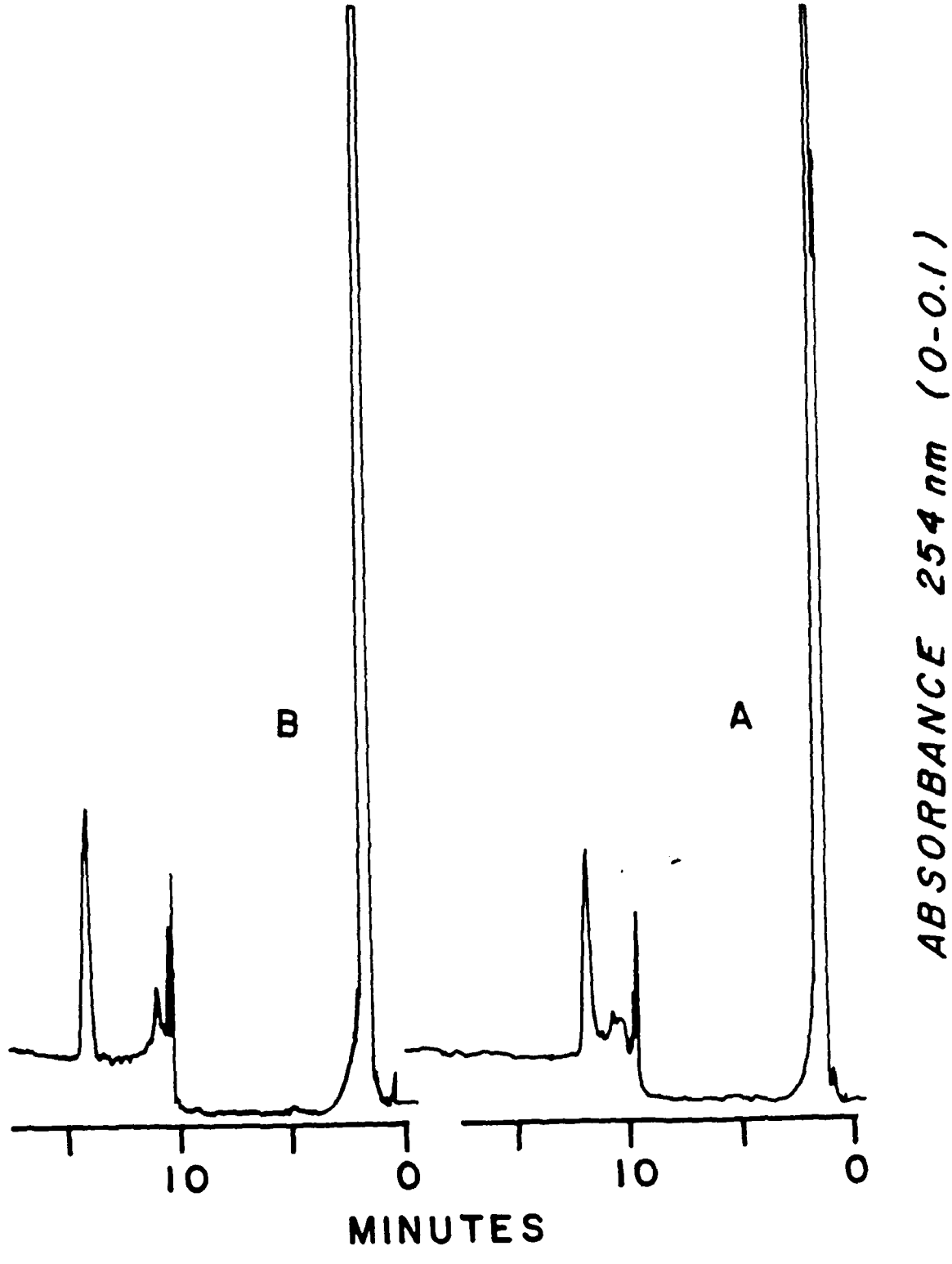


Figure 3. HPLC Profiles of the Ethyl Acetate Extracts of Feces. Chromatogram A=CT 3^2; chromatogram B=BC 18^1. Samples were run as described in Figure 1.

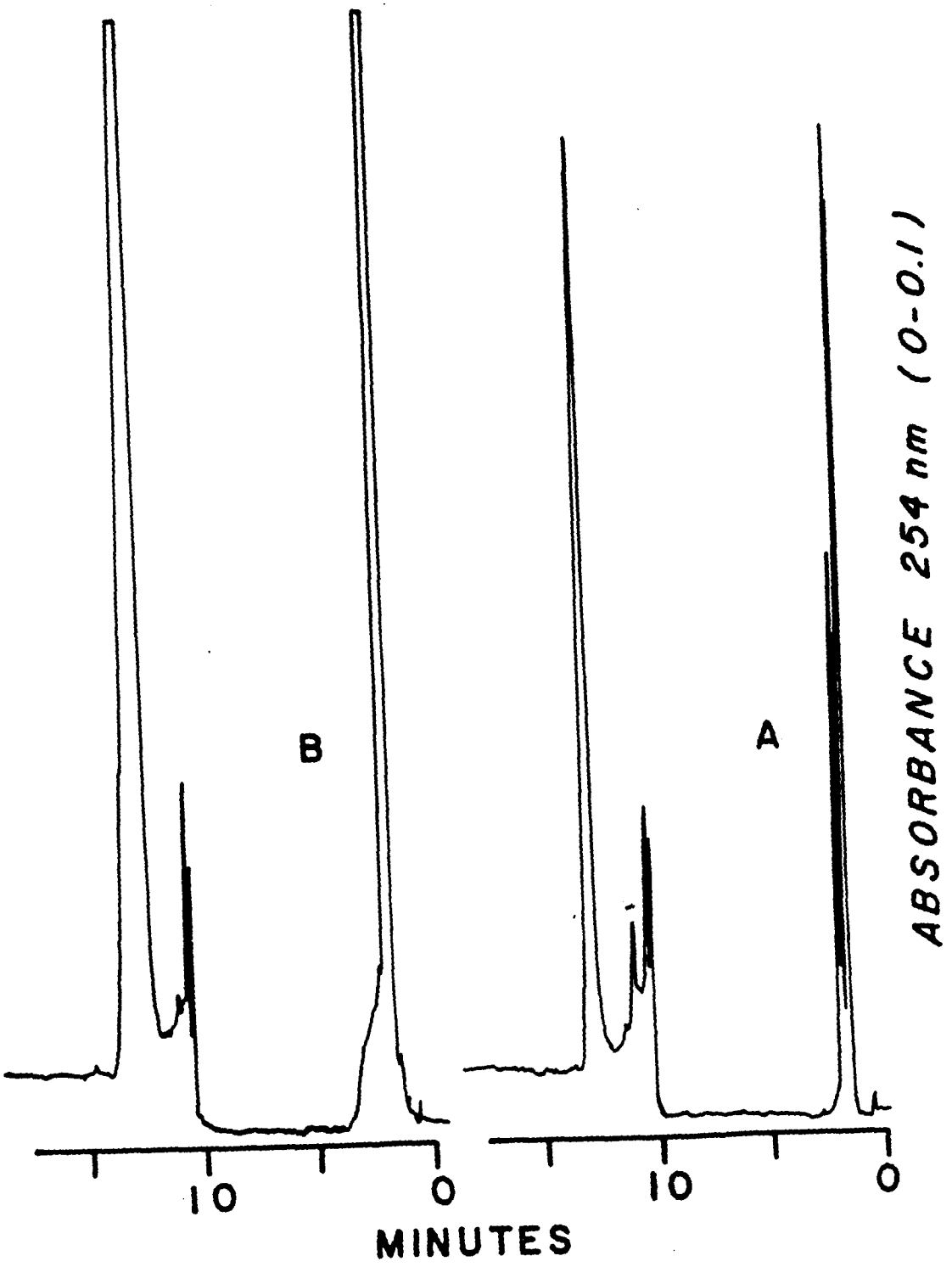
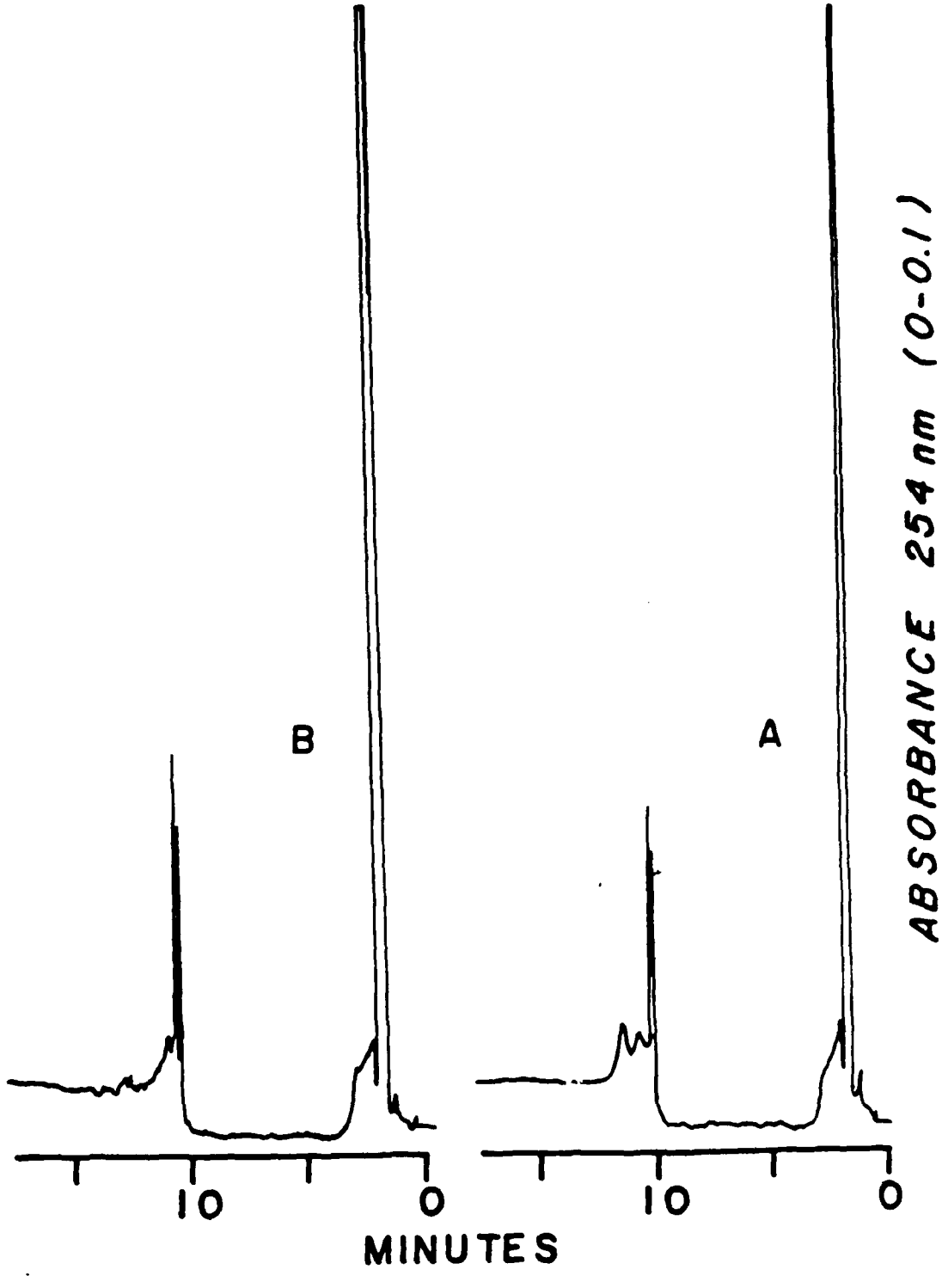


Figure 4. HPLC Profiles of the Acetonitrile Extracts of Feces. Chromatogram A=CT 3[2]; chromatogram B=BC 18[1]. Samples were run as described in Figure 1.



4. **Tetrahydrofuran Extracts**

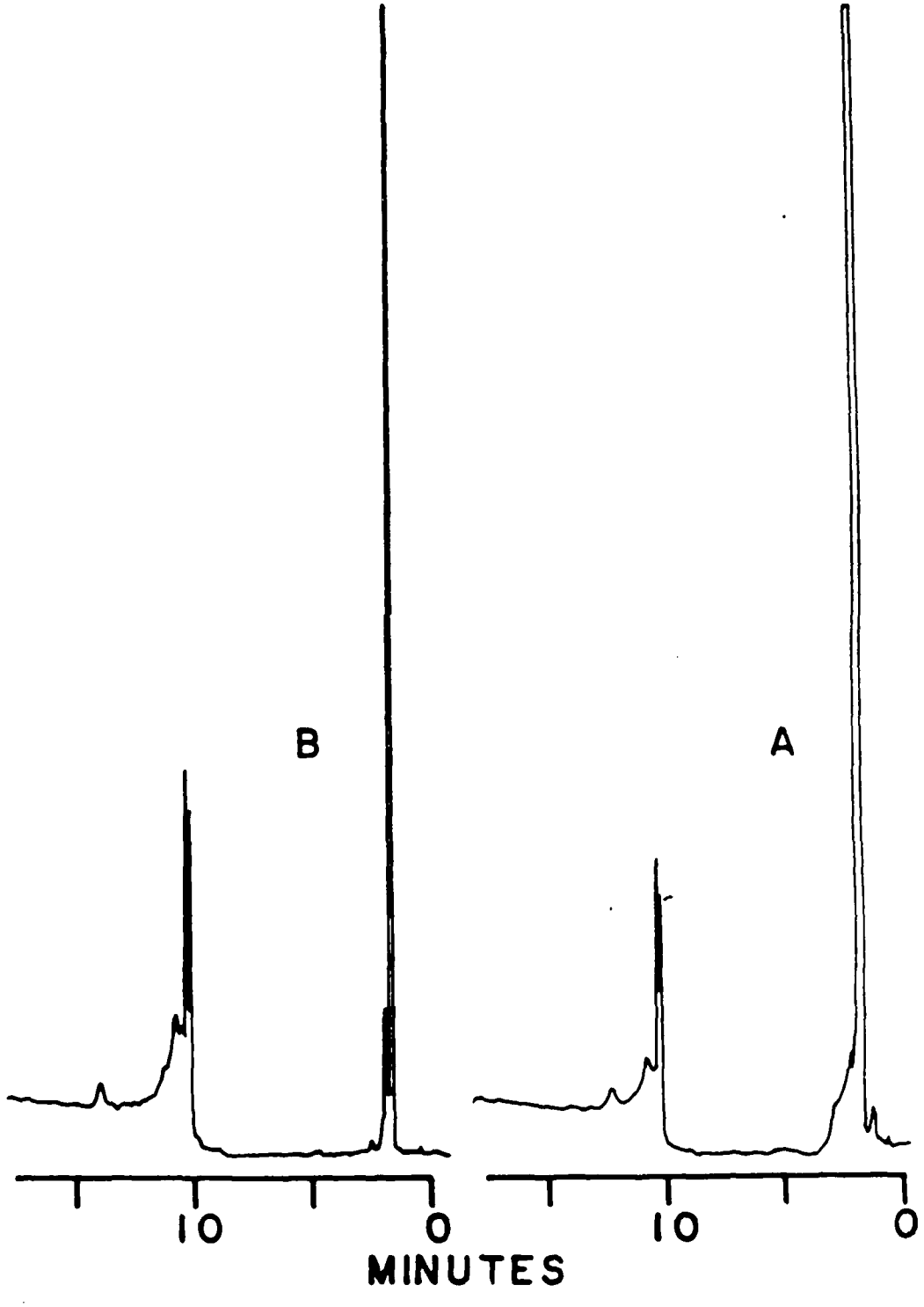
The HPLC profiles of 25 µl of the tetrahydrofuran solutions of these extracts of CT 3^2 and BC 18^1 are shown in Figures 5A and 5B, respectively. Both chromatograms appear to contain metabolites, but the TLC results indicated that only the CT 3^1 contained metabolites.

Discussion

The results, TLC and HPLC, of this experiment indicate the developed procedures give consistent results between the two methods. Furthermore, the preliminary HPLC method shows promise for development into an isocratic method of eluting the parent and metabolites from sample extracts. A probable solvent composition would be 60% methanol- 40% methylene chloride. The HPLC method is also amiable to scale - up to the preparative scale, allowing for component collection to quantitate recovery by LSC and leading to the isolation/identification of metabolites of WR-158,122.

Other HPLC experiments are in progress. These include a similar preliminary examination of the rat urine extracts (interim report 3) and examination of the 24, 48 and 72 hr samples. HPLC examination of bile extracts has not yet begun.

Figure 5. HPLC Profiles of the Tetrahydrofuran Extracts of Feces Chromatogram A=CT 3^2; chromatogram B=BC 18^1. Samples were run as described in Figure 1.



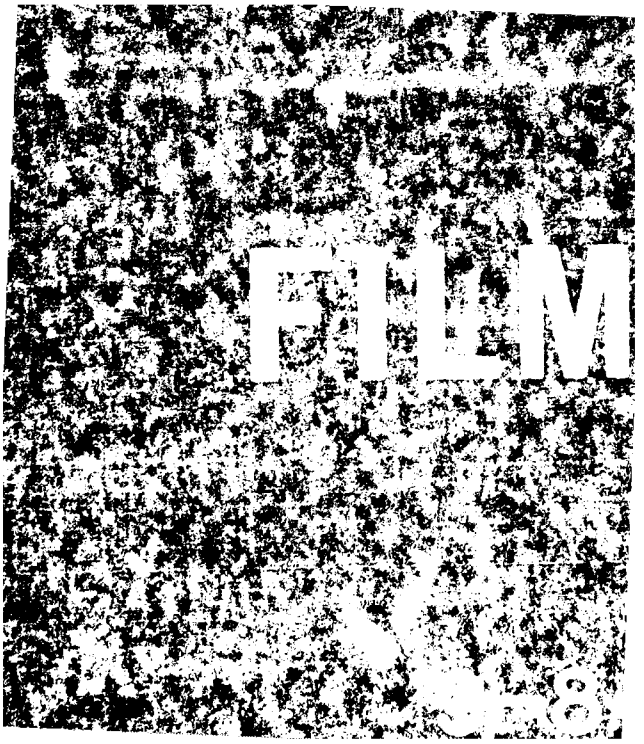
Conclusions

1. A convenient preliminary HPLC method has been developed for the examination of rat feces extracts for similarities and differences.

2. Ethyl acetate was found to extract essentially all of the parent compound from both BiDuCa and CT rat feces.

3. Metabolites of WR-158,122-^{14}C have been observed by both HPLC and TLC.

4. Scale-up of HPLC methods for metabolite isolation/identification appears to be a realistic and logical next direction.


FILM